

## Minireview

## MicroRNA function in animal development

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**Abstract** MicroRNAs (miRNAs) are small non-coding RNA molecules that post-transcriptionally regulate gene expression by base-pairing to mRNAs. Hundreds of miRNAs have been identified in various multicellular organisms and many miRNAs are evolutionarily conserved. Although the biological functions of most miRNAs are unknown, miRNAs are predicted to regulate up to 30% of the genes within the human genome. Gradually, we are beginning to understand the functions of individual miRNAs and the general function of miRNA action. Here, we review the recent advances in miRNA biology in animals. Particularly, we focus on the roles of miRNAs in vertebrate development and disease.

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**Keywords:** MicroRNA; Vertebrate; Development; Differentiation; Disease

## 1. Introduction

The founding microRNA, *lin-4*, was first thought to be an isolated case. The *lin-4* gene was identified by genetics in the nematode *Caenorhabditis elegans* (*C. elegans*) and is involved in developmental timing. Surprisingly, this gene does not encode a protein but codes for two small RNA molecules of ~60 and ~22 nucleotides in length [1]. The former is the precursor of the smaller and can form a secondary stem-loop structure that is phylogenetically conserved in nematodes. The smaller RNA molecule regulates the *lin-14* gene by base-pairing to imperfect complementary sites in the 3' untranslated region (UTR) of *lin-14* mRNA [2]. This form of post-transcriptional gene silencing by a small regulatory RNA was initially regarded as a rare phenomenon, specific for nematodes. However, the discovery of *let-7* changed this idea. *Let-7* is another small RNA that is also involved in developmental timing in *C. elegans* and regulates the *lin-41* gene [3,4]. Hence, *lin-4* and *let-7* were both called small temporal RNAs (stRNAs) for their role in developmental timing [5]. In contrast to the *lin-4/lin-14* pair, *let-7* and *lin-41* are evolutionarily conserved among a wide variety of multicellular organisms. Furthermore, *let-7* is also differentially expressed during development of various bilaterian animals [5]. This discovery implied that

regulation of gene expression by these small RNAs was not restricted to nematodes and could be a general mechanism present in many multicellular organisms. Indeed, almost one hundred of such small regulatory RNAs were readily identified in *C. elegans*, *Drosophila* and human [6–8]. Since most of these newly identified small RNAs are not expressed in a temporal-specific manner, as *lin-4* or *let-7*, but in a spatial- or tissue-specific manner, the small regulatory RNAs are now called microRNAs (miRNAs). Currently, miRNAs have been identified in many other multicellular organisms, and it is estimated that vertebrate genomes may encode several hundred or perhaps up to 1000 different miRNAs [9,10], which may regulate up to 20–30% of the genes [10,11]. Here, we review the recent advances in miRNA identification, expression and function in animals. Particularly, we focus on the roles of miRNAs in vertebrate development and disease. A similar review on plant miRNAs can be found elsewhere in this issue of FEBS Letters [12].

## 2. Identification and expression of miRNAs

### 2.1. Identification of miRNAs

Currently, 2909 miRNA genes have been annotated in miR-Base (release 7.0) [13]. To annotate a newly identified small RNA as miRNA it must fulfill several criteria. First, miRNAs are expressed as ~22-nucleotide RNA molecules. Second, miRNAs are encoded in the genome as phylogenetically conserved hairpin structures with a low free energy. Third, miRNA expression levels are reduced in animals defective in miRNA biogenesis [14]. It is worth noting that not all miRNAs exactly fit these criteria. In particular, the requirements for annotation of closely related miRNAs in miRNA families or other species is less stringent [14]. Members of the miRNA gene class in animals are identified in several ways (Fig. 1).

The founding members, *lin-4* and *let-7*, were both identified in *C. elegans* based on their mutant phenotypes in forward genetic screens [1,3]. Other forward genetic screens have only led to the identification of one additional miRNA in *C. elegans* (*lgy-6* [15]) and two additional miRNAs in *Drosophila* (*bantam* and *miR-14* [16,17]). Although small in number, these five miRNAs are probably the best-studied miRNAs so far. The unbiased nature of forward genetics screens can discover miRNAs that cannot easily be detected by any other method. For example, the *lgy-6* miRNA could not be identified by either cDNA cloning nor computational predictions [15]. On the other hand, many miRNAs are likely to be missed in forward genetic screens because miRNA genes are relatively

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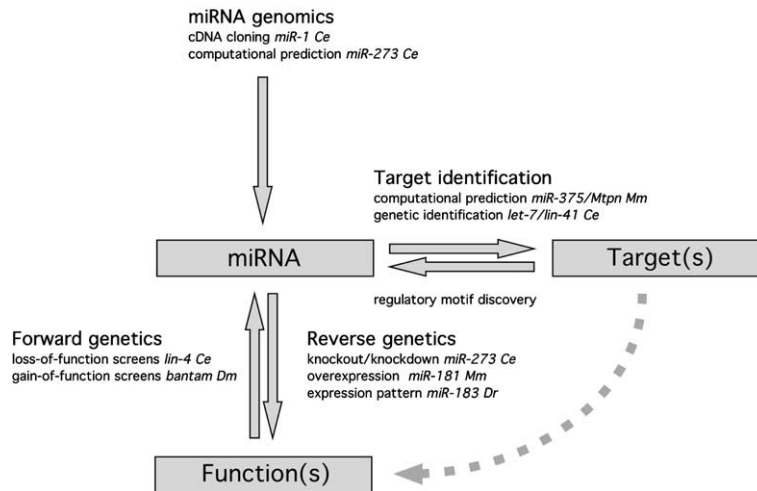


Fig. 1. Approaches for the identification of miRNA genes, target genes and functions of miRNAs in animals. For each approach an example miRNA is given that is mentioned in the text or literature. Abbreviations. Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio* (zebrafish); Hs, *Homo Sapiens*; Mm, *Mus musculus*.

small genes (about 50 times smaller than the average protein coding gene), which are not often hit by the classical mutagens. In addition, many miRNAs might have redundant functions and therefore give only very subtle or no phenotypes when mutated [18].

To bypass the rare genetic discovery of miRNAs, several labs identified more than a hundred miRNAs by cDNA cloning and subsequent sequence analysis [6–8,19]. This approach has the advantage that it can be applied to any organism, even if little or no genomic information is available. In addition, miRNAs can be identified independent of their function, thus also allowing the identification of redundant miRNAs. By cDNA cloning, miRNAs have now been identified in diverse animals like nematodes [6,8,20], flies [7,21], mammals [7,22–28], frogs [29], fish [30,31] and several mammalian viruses [32,33].

One limitation of the cloning strategy is that there is a potential bias in cloning small RNA molecules. Highly expressed miRNAs or technically easy to clone miRNAs might overshadow the miRNAs that are only expressed at particular developmental stages, under specific circumstances, in specific cell types or which are otherwise difficult to clone. To overcome such a bias, several computational algorithms have been developed to predict miRNAs in nematode, fly and vertebrate genomes [20,30,34–38]. These approaches use genome comparisons to search for evolutionary conserved hairpin structures, which could encode precursor miRNAs (pre-miRNAs). Together, these algorithms predict that there are ~110 miRNAs in the *Drosophila* genome, ~120–300 miRNAs in the *C. elegans* genome, and ~255 miRNAs in vertebrate genomes such as human [20,30,34–37]. However, more recently three independent computational approaches indicate that the actual number of miRNAs in vertebrates might be much higher [9,10,39]. We found, by phylogenetic shadowing of primate miRNAs, that there is a high degree of conservation in miRNA regions, but relatively low conservation in sequences immediately flanking these regions (see Fig. 2A for example). This feature was used to predict over 800 new miRNAs in mammalian genomes of which many are conserved in other vertebrate genomes. The expression of some (16) of these predicted miRNAs could be confirmed experimentally [9].

Xie and coworkers searched for conserved motifs in 3' UTRs by systematic comparison of several mammalian genomes. More than half of the recovered motifs correspond with the seed sequences (nucleotide positions 2–8) of known miRNAs. The remaining motifs were used to predict 129 new miRNAs [10]. Finally, Bentwich et al. [39] used an integrative approach, combining bioinformatic predictions with microarray analysis and sequence-directed cloning, to identify 89 new miRNAs and predict the total number of human miRNAs to be at least 800. Together, these studies predict that the total number of miRNAs in vertebrate genomes might be doubled and maybe approach as many as 1000 [9]. These candidate miRNAs require further experimental verification.

## 2.2. Expression of miRNAs

The distribution of miRNA genes within genomes is not random. More than half of known mammalian miRNA genes are within introns of host genes [40]. Microarray analysis indicates that many of these miRNAs are coexpressed with their host genes [41]. The location of some of these intronic miRNAs is evolutionarily conserved and they are similarly coexpressed with their host genes in different animals. For example, the *mir-126* gene is located in an intron of the *EGFL7* gene of mouse, human [40] and zebrafish. *mir-126* and *EGFL7* are similarly expressed in endothelial cells of the heart and blood vessels in both humans and zebrafish [41,42]. Such homologies suggest important and evolutionarily conserved roles for these miRNAs. Approximately 40% of human miRNA genes are in genomic clusters. Many of these miRNAs are also coexpressed [41], which could have biological advantages. Finally, miRNA genes are frequently located at fragile sites and genomic regions involved in cancers [43], suggesting that these miRNAs are involved in cellular processes impaired in cancers, such as cell growth, cell division and proliferation.

miRNAs have a wide variety of expression patterns. In *C. elegans* and *Drosophila* some miRNAs are differentially expressed in time during development, whereas others seem to be more ubiquitously expressed [5–8,20,21,34,37,44]. For most of these miRNAs only the temporal expression patterns have been determined. However, reporter constructs show that some miRNAs are only expressed in specific cell types [15,45].

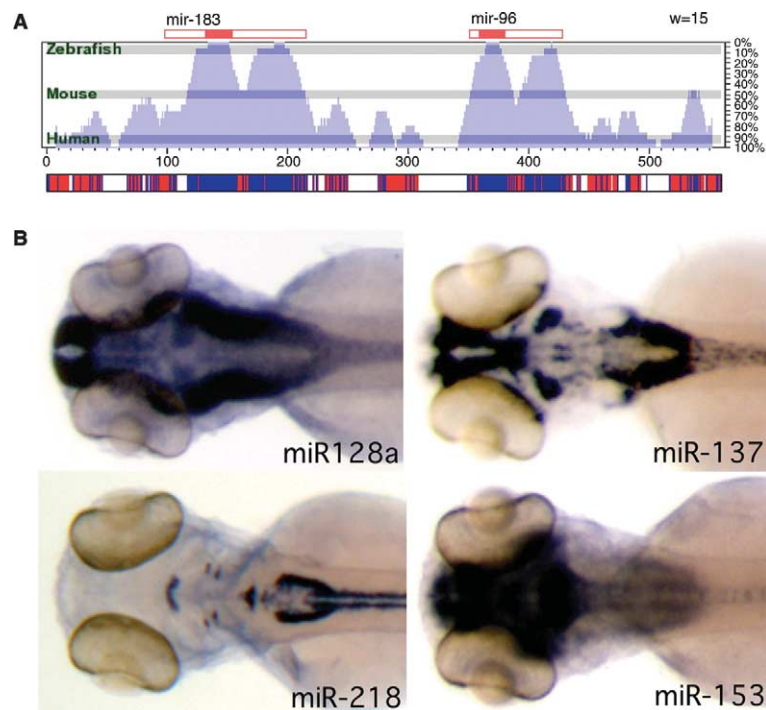


Fig. 2. Vertebrate miRNAs in zebrafish. (A) Phylogenetic conservation of two clustered vertebrate miRNA genes. miRNA regions (indicated by the red boxes) are more conserved than flanking sequences. See [9] for details. (B) Example expression patterns of conserved brain-specific miRNAs in zebrafish embryos. Adapted with permission from [42].

The tissue-specific miRNA expression has been best-studied in vertebrates. First, the cloning frequency from particular tissues or cells is different for many miRNAs. For example, several miRNAs are predominantly cloned from mouse heart, liver or brain tissues [23], embryonic stem cells [27,28] or pancreatic islet cells [46]. Further analysis by northern blots or microarrays reveals that many other vertebrate miRNAs are also tissue-specifically expressed [41,42,47–51]. Our recent data from in situ hybridizations in zebrafish embryos indicate that ~80% of the conserved vertebrate miRNAs that are expressed during embryonic development are tissue-specific. This tissue-specificity is not restricted to only a few major organs, but virtually all zebrafish tissues and even individual cell types within tissues have specific expression of one or a few miRNAs. For example, miR-183 is specifically expressed in the hair cells of sensory epithelia [42]. This high number of tissue-specific miRNA expression patterns suggests that these miRNAs have a role in tissue differentiation.

Many miRNAs are similarly expressed in mammals and zebrafish, indicating an evolutionarily conserved function. For example, many miRNAs that are brain-specific in mammals have very distinct in situ expression patterns in the brain of zebrafish embryos (e.g., see Fig. 2B [42]). In addition, these miRNAs are also differentially expressed during mammalian brain development [26,52], suggesting a conserved role in vertebrate brain function.

### 3. miRNA biogenesis and mechanism

The mammalian miRNA biogenesis pathway (and that of other animals) can be divided into multiple steps (Fig. 3). Ini-

tially, miRNA genes are transcribed by RNA polymerase II into long primary miRNAs (pri-miRNAs) [53,54]. The processing of these pri-miRNAs into the final mature miRNAs occurs stepwise and compartmentalized [55]. pri-miRNAs are processed in the nucleus into ~70–80-nucleotide precursor miRNAs (pre-miRNAs) by the RNase III enzyme Drosha [56]. Drosha forms a microprocessor complex with double-stranded RNA-binding protein DGCR8 (Pasha in flies) [57–60]. pre-miRNA hairpins are exported from the nucleus by Exportin-5 in the presence of Ran-GTP as cofactor [61–63]. In the cytoplasm, the pre-miRNAs are processed into ~22-nucleotide duplex miRNAs by the RNase III enzyme Dicer [64–66]. Dicer was originally discovered by its role in RNA interference (RNAi) in which it processes long double-stranded RNA into small interfering RNAs (siRNAs) that mediate RNAi [67–70]. Dicer interacts with the double-stranded RNA-binding protein TRBP (RDE-4 in *C. elegans* and Loquacious in *Drosophila* [71–75]), which likely bridges the initiator and effector steps of miRNA action. Next, miRNA duplexes are unwound, which starts at the duplex end with the lowest thermodynamic stability. The miRNA strand that has its 5' terminus at this end is the future mature miRNA (also called guide RNA) [76–78].

The mature miRNAs are incorporated into a ribonucleoprotein complex, miRNP [25,79], which is similar, though not necessarily identical, to the RNA-induced silencing complex (RISC), the effector of RNAi [80]. In RISC, miRNAs can mediate downregulation of target gene activity by two modes: translational inhibition or target mRNA cleavage (Fig. 3). The choice is made based on the degree of complementarity between the miRNA and target gene in combination with an Argonaute family protein. Near-perfect complementarity results in cleavage, followed by general RNA degradation of

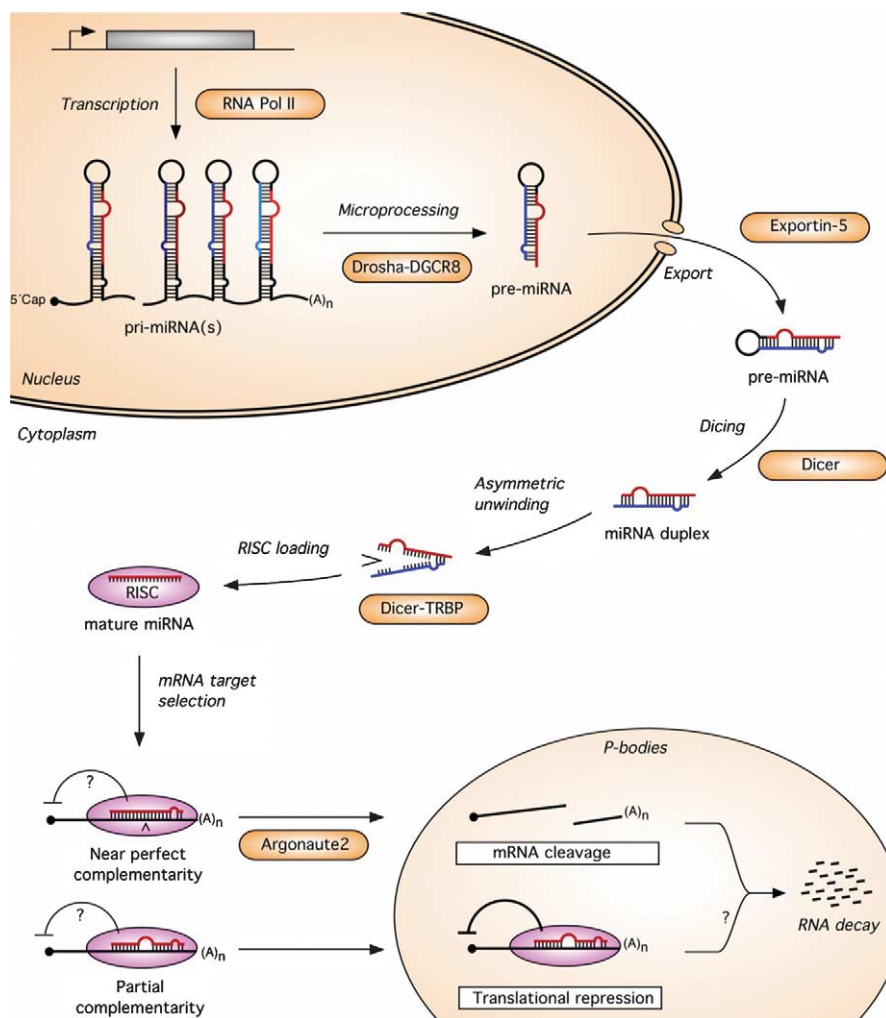


Fig. 3. A model for the miRNA biogenesis pathway and mechanism in mammals. See text for details.

the targets, whereas partial complementarity causes translational inhibition [79,81–84]. The exact mechanism for translational inhibition is not known. miRNA-targeted mRNAs could, for example, be sequestered on polysomes [85,86] or be recruited to P-bodies where they are depleted of the translation machinery and eventually degraded [87,88]. In contrast to plants, miRNA-directed cleavage in animals (such as the cleavage of *HOXB8* mRNA by miR-196 in mouse embryos [89,90]) is less common than translational inhibition. Argonaute proteins are prominently present in RISC and all mammalian Argonaute proteins bind miRNAs and siRNAs. However, in both mammals and *Drosophila* Argonaute2 (eIF2C2) is the sole catalytic engine of RISC that mediates targeted RNA cleavage [70,91–93]. Furthermore, *Drosophila* Argonaute1 and the two Argonaute1 homologs *alg-1* and *alg-2* in *C. elegans* are required for miRNA production, stability and function [65,94].

#### 4. Biological functions of miRNAs in animals

miRNAs have diverse biological functions (Table 1). Clues about the function of miRNAs in animals have been obtained by several approaches (Fig. 1). First, several miRNAs were identified by loss- and gain-of-function genetic screens in

*C. elegans* and *Drosophila*. Since these miRNAs were found based on their biological functions, their described functions are likely the most accurate. Other genetically assigned miRNA functions come from reverse genetic approaches. These approaches include miRNA knockout or knockdown [95–98] and miRNA overexpression studies. Furthermore, the miRNA expression profiles, determined by, for example, microarray- and in situ analysis, have revealed specific miRNA expression patterns, which give hints to the functions of specific miRNAs.

miRNAs function through the regulation of target genes. Only a few (seven) of the miRNA target genes have been identified genetically by forward and reverse genetic screens. Most miRNA target genes come from computational predictions. However, only a handful of these predicted targets have been experimentally validated in *Drosophila* and human cells using reporter constructs and the majority remains to be verified [38,99]. Recent miRNA target predictions indicate that for each *Drosophila* miRNA there are on average ~100 different target genes of which most are regulated through the seed site interactions [100]. miRNA target predictions in mammals indicate that ~10–30% of the genes might be under control of the currently known miRNAs [10,11,101] and that each mammalian miRNA regulates on average ~200 target genes through

Table 1  
Biological functions of miRNAs in animals and disease

miRNA	Target(s)	Function(s)	References
<i>Ceanorhabditis elegans</i>			
<i>lin-4</i>	<i>lin-14</i> , <i>lin-28</i>	Early developmental timing	[1,106,107]
<i>let-7</i>	<i>lin-41</i> , <i>hbl-1</i> , <i>daf-12</i> <i>pha-4</i> , <i>ras</i>	Late developmental timing	[3,4,108–111]
<i>lsy-6</i>	<i>cog-1</i>	Left/right neuronal asymmetry	[15]
<i>miR-273</i>	<i>die-1</i>	Left/right neuronal asymmetry	[112]
<i>Drosophila melanogaster</i>			
<i>bantam</i>	<i>hid</i>	Programmed cell death	[16]
<i>miR-14</i>	<i>Drice?</i>	Programmed cell death and fat metabolism	[17]
<i>miR-7</i>	Notch targets?	Notch signaling	[113,115]
<i>Danio rerio</i>			
<i>miR-430</i>	?	Brain morphogenesis	[120]
<i>Mus musculus</i>			
<i>miR-196</i>	<i>Hoxb8</i>	Developmental patterning	[90]
<i>miR-181</i>	?	Hematopoietic lineage differentiation	[47]
<i>miR-1</i>	<i>Hand2</i>	Cardiomyocyte differentiation and proliferation	[122]
<i>miR-375</i>	<i>Mtpn</i>	Insulin secretion	[46]
<i>Human and other vertebrate cell lines</i>			
<i>miR-16</i>	Several	AU-rich element-mediated mRNA instability	[130]
<i>miR-32</i>	Retrovirus <i>PFV-1</i>	Antiviral defense	[97]
<i>miR-143</i>	<i>Erk5?</i>	Adipocyte differentiation	[123]
<i>SVmiRNAs</i>	<i>SV40</i> viral mRNAs	Susceptibility to cytotoxic T cells	[145]
<i>Cancer in humans</i>			
<i>miR-15-miR-16</i>	?	Downregulated in B-cell chronic lymphocyte leukemia	[137]
<i>miR-143</i> , <i>miR-145</i>	?	Downregulated in colonic adenocarcinoma	[138]
<i>miR-155/BIC</i>	?	Upregulated in diffuse large B-cell lymphoma	[139,140]
<i>let-7</i>	<i>Ras?</i>	Downregulated in lung cell carcinoma	[111,141]
<i>miR-17-92</i>	?	Upregulated in B-cell lymphoma	[142]

interaction between the seed sequence and the complementary target site [102]. Many genes have several target sites for either one miRNA or a few different miRNAs. In some cases, multiple target sites are essential for proper regulation in vitro or in vivo [100,102–105]. The combinatorial regulation by different miRNAs adds another layer of complexity that can result in more fine-tuned activity in diverse cell-types, similar to the combinatorial regulation by different transcription factors.

#### 4.1. Developmental timing in worms

The two best-studied miRNAs, *lin-4* and *let-7*, were found by forward genetics to act in the heterochronic pathway to regulate developmental timing in *C. elegans* [1,3]. *Let-7* was identified as a suppressor of *lin-14* mutants [3], indicating that they act in the same pathway. Loss-of-function mutations in *lin-4* and *let-7* both cause retarded development but at different developmental stages. Whereas *lin-4* null mutants reiterate specific fates of the first larval stage at subsequent later stages, *let-7* null mutants reiterate larval cell fates at the adult stage. Conversely, overexpression of *let-7* gives precocious development, the opposite heterochronic phenotype [3]. The timing of the phenotypes corresponds with the onset of *lin-4* and *let-7* expression, which is early and late in development, respectively [1,3]. Thus, *lin-4* is an early developmental timer and *let-7* a late developmental timer.

Two *lin-4* targets, *lin-14* and *lin-28*, were both identified as genes that act downstream of *lin-4* in the heterochronic pathway. Loss-of-function mutations in these genes cause phenotypes that are opposite to that of *lin-4* mutants: precocious cell fates of late developmental stages during the first

and second larval stages [2,106]. Furthermore, gain-of-function mutations in the *lin-14* 3' UTR cause a phenotype similar to *lin-4* loss-of-function mutations [107]. *Lin-14* encodes a nuclear protein, which regulates the transition from the first to the second larval stage [2]. *Lin-28* encodes a cold-shock zinc finger protein that promotes the transition of the second to third larval stage [106]. Regulation by *lin-4* might not be restricted to the early stages since it is also required for downregulation of a *hbl-1::GFP* reporter gene, which has target sites for both *lin-4* and *let-7* in its 3' UTR, in ventral nerve cord neurons of adults [108].

Multiple genes are regulated by *let-7* in tissue-specific manners. The *lin-41* target gene was identified in a genetic screen as strongest suppressor of the lethality of *let-7* mutants. It encodes a RBCC (ring finger, B box, coiled coil) protein. Null mutations in *lin-41* cause the opposite phenotype to that of the *let-7* mutants, namely precocious expression of adult fates at larval stages [3,4]. Overexpression of wild-type *lin-41* results in reiteration of larval fates, similar to *let-7* loss-of-function mutants [4]. *Lin-41* is partially redundant with the hunchback-like gene *hbl-1* in the hypodermal seam cells and negatively regulates the timing of the adult specification transcription factor LIN-29 [108,109]. The defects in *let-7* mutant strains are also suppressed by loss of *hbl-1* function. *Hbl-1* is involved in timing events in neurons of the ventral nerve cord and vulval cells [108,109]. Several other targets for *let-7* have been identified in an RNAi knockdown screen for genes that have *let-7* target sites and suppress *let-7* mutant lethality. This yielded the nuclear hormone receptor *daf-12*, which is another target in seam cells of the hypodermis and

the forkhead transcription factor *pha-4*, which is an intestinal target. It also identified the zinc finger transcription factor *die-1*, the putative chromatin remodeling factor *lss-4* and the RAS ortholog *let-60* as probable targets [110]. In addition, recent experiments indicate that *let-60/RAS* is regulated by *let-7* in the hypodermal cells and by the *let-7* family member miR-84 in vulval cells [111].

#### 4.2. Neuronal left/right asymmetry in worms

A cascade of genes, involving two miRNAs, determines the left/right asymmetric expression of chemosensory receptor genes in the left (ASEL) and right (ASER) chemosensory neurons of *C. elegans* [15,112]. The *lgy-6* miRNA is expressed in the ASEL neuron and inhibits the expression of its target, the Nkx-type homeobox gene *cog-1*. This ultimately leads to the expression of the GCY-7 chemosensory receptor in ASEL [15]. In the ASER neuron, miR-273 inhibits the translation of *die-1* mRNA. DIE-1 is a zinc-finger transcription factor needed for the transcription of *lgy-6*. Therefore, the expression of miR-273 leads to the downregulation of *lgy-6* and subsequently to the expression of the GCY-5 chemosensory receptor in ASER [112]. Thus, inverse and sequential expression of two miRNAs leads to asymmetric expression of chemosensory receptors in neurons of *C. elegans*.

#### 4.3. Programmed cell death in flies

In *Drosophila*, two miRNAs, *bantam* and miR-14, were genetically found to be involved in the regulation of programmed (apoptotic) cell death. The *bantam* miRNA was identified in a gain-of-function EP element insertion screen for genes that affect cell proliferation. Overexpression of *bantam* causes tissue overgrowth and inhibits proliferation-induced apoptosis [16]. In contrast, loss of *bantam* function is lethal. *Bantam* negatively regulates Hid protein expression in vivo and thereby blocks *hid*-induced apoptosis in the eye [16]. miR-14 was identified by a genetic screen for inhibitors of programmed cell death. Animals with miR-14 loss-of-function alleles have enhanced Reaper-induced cell death, whereas overexpression of miR-14 suppresses cell death. The absence of miR-14 is also characterized by semilethality, reduced lifespan and stress sensitivity. Furthermore, miR-14 is involved in the regulation of fat metabolism in a dose-dependent manner [17]. A potential target for miR-14 is the apoptotic effector caspase Drice. Drice is upregulated in the absence of miR-14, suggesting that the *drice* mRNA is regulated, either directly or indirectly, by miR-14 [17]. Other miRNAs that potentially act in the programmed cell death pathway are miR-2 and miR-13. They are predicted to regulate the proapoptotic genes *reaper*, *grim* and *sickle* [113]. miR-2 downregulates reporter constructs with the 3' UTRs of these target genes in vitro and in vivo [113]. Furthermore, knockdown of miR-2 or miR-13 may give developmental defects [114].

#### 4.4. Notch signaling in flies

Several miRNAs may be involved in regulating the Notch signaling pathway. Notch signaling is essential for proper patterning and development of all multicellular organisms. In *Drosophila*, the Notch target genes, encoding basic helix–loop–helix (bHLH) repressors and Bearded family proteins, are post-transcriptionally regulated by different combinations of the K-box, GY-box and Brd-box motifs in their 3' UTRs.

These 6–7 nt motifs are evolutionary conserved [113,115] and are complementary to the seed sequences of several miRNAs [116]. Reporter constructs with the 3' UTRs of the bHLH and Bearded *Notch* target genes are downregulated by multiple members of miRNA families in vivo [113,115]. The individual K-boxes, GY-boxes or Brd-boxes are necessary and sufficient for this miRNA-mediated regulation. The K-box is regulated by miR-2 and miR-11, the GY-box by miR-7, and the Brd-box by miR-4 and miR-79 [115]. Ectopic overexpression of miR-7 or a cluster of K-box-regulating miRNAs leads to reduced expression of downstream notch targets such as Cut. Furthermore, it causes notching of the wing margin, reduced wing vein spacing, thickening of the wing veins and ectopic microchaete bristles [113,115]. These phenotypes are characteristic of reduced Notch signaling, supporting the idea that the Notch signaling pathway is regulated by miRNAs.

#### 4.5. Early vertebrate development

Several observations show that miRNAs are essential for the normal development of mammals. First, mouse and human ES cells express a specific set of miRNAs that are downregulated upon differentiation into embryoid bodies [27,28]. Second, ES cells that are deficient in *dicer* are viable, but do not form mature miRNAs and they fail to differentiate in vitro and in vivo [117]. Third, *dicer* mutant mouse embryos die before axis formation during gastrulation and have ES cell loss [118].

To study the global role of miRNAs in early embryonic development we knocked out the *dicer* gene in zebrafish [119]. Zebrafish embryos that are *dicer* mutant develop normally but arrest ~8 days after fertilization when they run out of maternal Dicer and are depleted of mature miRNAs. At this point all the major organs have been formed. This arrest suggests that miRNAs are essential for development and growth of tissues beyond this stage. Knockdown of maternal Dicer mRNA by morpholinos results in an earlier arrest and delay in miRNA production. However, during the first 24 h, these embryos still develop quite normally, which indicates that miRNAs are not essential for earliest developmental processes [119]. To also exclude a role for maternal Dicer protein Giraldez et al. [120] generated maternal-zygotic mutants from the *dicer* knockout zebrafish. As expected, these maternal-zygotic *dicer* mutants do not process pre-miRNAs into mature miRNAs. Surprisingly, they have only some mild defects during early development. They have intact axis formation and cell regionalization and differentiate into multiple cell types and tissues, indicating correct patterning. However, they show morphogenesis defects during gastrulation, brain formation, neural differentiation, somitogenesis and heart development. Together, this implies that miRNAs are not essential for cell fate determination and early patterning, but are essential for subsequent later steps in embryonic development [120]. This notion is further supported by the temporal and spatial expression patterns of conserved vertebrate miRNAs in zebrafish embryos [31,42]. The majority of these miRNAs are not expressed early, but show highly tissue-specific expression during the later stages [31,42], suggesting that their role is not in tissue fate establishment but in differentiation or maintenance of tissue identity [31,42]. Interestingly, *dicer* mutant primordial germ cells give rise to viable germ lines in both male and female zebrafish, which can form embryos. This indicates that

mature miRNAs are also not cell-autonomously required in gametes during germ-line development in zebrafish [120].

Why do *dicer* mutant mouse embryos without mature miRNAs die before axis formation while zebrafish *dicer* mutant embryos without mature miRNAs survive that stage? This may be explained by the differences in dynamics during early embryonic development. Compared to mouse development zebrafish early development is extremely rapid. One day after fertilization mouse embryos have undergone cell division once. During the same period zebrafish embryos have established a basic body plan and have formed all major organs. Maybe most miRNAs are not produced fast enough, miRNA levels are too low or miRNA action is too slow to function in the rapid dividing cells of early zebrafish embryos. Alternatively, other RNAi-related mechanisms that are controlled by Dicer, such as the formation of heterochromatin structures and centromeric silencing [117,121], may have a function in early development of mice, but not in zebrafish.

The only miRNAs that are highly expressed during early zebrafish development are members of the miR-430 miRNA family. The miR-430 miRNA family is expressed from a genomic cluster of more than 90 copies within 120 kb, is conserved in other fish species and related miRNAs are present in mammals and frogs [29,31,120]. In frogs, these related miRNAs are also expressed early in development [29]. Injection of miR-430 into maternal-zygotic *dicer* mutant zebrafish embryos rescues the brain morphogenesis defects and to some extent the other neuronal defects, indicating that the miR-430 miRNA family regulates neurogenesis in zebrafish [120].

#### 4.6. Late vertebrate development

Some other miRNAs have a more specialized function at later stages of vertebrate development (Table 1). First, miR-196 is involved in HOX gene regulation. The *mir-10* and *mir-196* genes of various vertebrates reside in the homeobox (HOX) clusters. Like the HOX genes, miR-10 and miR-196 are colinearly expressed in time and space along the anterior-posterior (head-to-tail) body axis [42,89]. In 15-day-old mouse embryos, miR-196 directs cleavage of *HOXB8* mRNA and probably also inhibits the *HOXC8*, *HOXD8* and *HOXA7* genes [90]. The expression of miR-196 is somewhat more posterior than the target HOX genes [89], which probably helps to define the posterior expression-boundary of the target HOX genes [90]. miR-10 may have a similar function in the HOX cluster since target sites for *mir-10* have also been predicted in HOX genes, for example in *HOXA3* [11]. Second, the muscle-specific miR-1 regulates the balance between differentiation and proliferation of cardiomyocytes during heart development in mice [122]. Overexpression of miR-1 in the heart of transgenic mice results in a proliferation defect and failure of ventricular cardiomyocyte expansion, indicative of premature differentiation of cardiomyocytes. Hand2, a transcription factor that promotes ventricular cardiomyocyte expansion is a target of miR-1. Hand2 is downregulated by overexpression of miR-1 in vivo, suggesting that it is a true target during mouse heart development [122]. Third, the miR-181 miRNA modulates hematopoietic lineage differentiation in mice. miR-181 is preferentially expressed in B-lymphocytes of mouse bone marrow and the thymus, the primary lymphoid organ [42,47]. Ectopic overexpression of miR-181 increases the fraction of B-lymphocytes and decreases the fraction of T-lymphocytes in vitro and

in vivo in mice. Although the target for miR-181 is not known, this indicates that miR-181 regulates mouse hematopoietic lineage differentiation [47]. Fourth, miR-143 regulates human adipocyte differentiation. miR-143 is strongly expressed in adipose (fat) tissue and is upregulated during the differentiation of human pre-adipocytes into adipocytes. Knockdown of miR-143 prevents adipocyte-specific gene expression and the accumulation of triglycerides but increases ERK5 protein levels [123]. ERK5 is a predicted target gene of miR-143 [124]. These data suggest that miR-143 is normally involved in promoting adipocyte differentiation or functioning, possibly through the regulation of ERK5 protein levels [123]. Finally, some miRNAs may have a role in imprinting during development. The *mir-127* and *mir-136* genes reside in the human imprinted 14q32 domain and are expressed from the maternally inherited chromosome, in the antisense orientation to a retrotransposon-like gene (*Rtl1*), which is exclusively expressed from the paternal chromosome. Lack of maternal gene expression in this region has been associated with abnormal development in mouse and humans [125,126].

#### 4.7. Physiological functions of vertebrate miRNAs

Several miRNAs do not have an obvious role in vertebrate development, but rather act in diverse physiological and cellular processes (Table 1). The miR-375 miRNA is specifically expressed in murine pancreatic islets cells, where it regulates the *Myotrophin* (*Mtpn*) gene and thereby glucose-stimulated insulin exocytosis [46]. Recently, it has been shown that miR-375 may act synergistically with miR-124a and let-7b [102]. Besides expression in the pancreatic islets, miR-375 is also highly expressed in the pituitary gland of zebrafish embryos [42]. This may indicate a role for miR-375 in the secretion of other hormones or neuroendocrine products, perhaps by regulating *Mtpn* in the pituitary gland. A role for miR-122a is the exclusion of cationic amino acid transporter (CAT-1) protein from the liver through translation inhibition and mRNA cleavage [127]. miR-122a is highly expressed in adult livers and its expression is upregulated during mammalian liver development. The expression pattern of CAT-1, a target of miR-122a, is inversely correlated with miR-122a expression and thus absent from the liver [23,127]. The functional consequence of this absence is however unknown. miR-122a may also have a role in mouse spermatogenesis. miR-122a is differently expressed in mouse testis during spermatogenesis and here probably regulates the target gene Transition protein 2 (*Tnp2*). *Tnp2* is a post-transcriptionally regulated testis-specific gene that is involved in chromatin remodeling during mouse spermatogenesis [128]. miRNA knockdown experiments indicate that several other miRNAs have roles in cell proliferation, cell growth and cell death in cell cultures [98,129]. For example, inhibition of miR-125b results in decreased proliferation of differentiated cancer cells [98]. Recently, it has been shown that miR-16 acts together with AU-rich element (ARE)-binding proteins in the degradation of ARE-containing mRNAs [130]. AREs are located in the 3' UTR of various short-lived mRNAs such as those encoding cytokines and proto-oncogenes. Rapid decay of reporter constructs containing these AREs requires proteins of the miRNA machinery. The human miR-16 has only limited complementarity to AREs. However, in the presence of the ARE-binding protein, tristetraprolin

(TTP), this limited complementarity is enough to destabilize ARE containing mRNAs. Knockdown and overexpression of miR-16 inhibits and promotes degradation of ARE containing mRNAs, respectively [130]. The miR-16-dependent regulation of ARE mRNAs by ARE-binding proteins suggests that we might expect several miRNAs to have a variety of potential activities as specificity factors for the interaction of mRNA regulatory proteins.

#### 4.8. General function of miRNAs

Thus far miRNAs have only been identified in multicellular organisms and are notably absent from unicellular organisms. This absence indicates that miRNAs, in general, may be essential for organisms to differentiate into multiple cell- and tissue types and/or to keep cells in a particular differentiation state. Several recent observations suggest that miRNAs are indeed such regulators of differentiation. First, undifferentiated or poorly differentiated cells do not require miRNAs to survive. This is apparent from the fact that mouse ES cells that do not form miRNAs are viable but fail to differentiate [117] and zebrafish germ cells do not need miRNAs for their survival and contribution to the germ line [120]. Second, most miRNAs are not expressed in early zebrafish development when cells are undifferentiated [31,42], but have highly tissue-specific expression at later stages when the most of cell types have been formed [42]. Third, in agreement with this absence, miRNAs are also not essential for tissue fate establishment during early zebrafish development, but are essential for later developmental steps [120] and tissue growth and/or functioning [119]. Fourth, many types of human cancer cells have reduced miRNA expression compared to their fully differentiated tissue of origin [131]. Fifth, vertebrate animals encode hundreds different miRNAs that are expected to regulate up to 30% of the genes [10,11]. Many of these miRNAs are widely conserved. Sixth, miRNAs have a high molecular abundance per cell. In *C. elegans* some miRNAs have been estimated to be present in as many as 50000 copies per cell, which is approximately 500-fold higher than the level of a typical worm mRNA [34]. Finally, transfection of the muscle-specific miR-1 and the brain-specific miRNA miR-124 into human HeLa cells shift the mRNA expression profile to that of muscle and brain cells, respectively [132].

From an evolutionary point of view, the regulation of gene expression by miRNAs may be an easy and flexible innovation, nevertheless crucial for cellular differentiation. The small size and simplicity of miRNA genes suggests that they can arise relatively easily and frequently de novo in animal and plants genomes. The independent creation of miRNAs is reflected by the high diversity between miRNA families and the lack of overlap between plant and animal miRNAs [133,134]. Furthermore, a few DNA base-pair changes corresponding to the seed sequences of miRNAs are likely to considerably alter the repertoire of target genes. In parallel, target genes can easily be subsumed under the control of miRNAs through changes of only a few base-pairs in the DNA corresponding to their 3' UTRs, similar to the acquisition of *cis*-regulatory sites in the promoters of genes. Single miRNAs are able to regulate about hundred target genes [132] and the combinatorial action of miRNAs is expected to regulate the expression of thousands of mRNAs [133]. The dampening of such a number of genes might be essential for

the initiation or maintenance of tissue differentiation [42,133]. In addition, the post-transcriptional regulation of gene expression might also dampen fluctuations in gene expression. This dampening results in stable protein levels, which might also be required to keep cells differentiated.

## 5. MicroRNAs and disease

The high number of miRNA genes, the diverse expression patterns and the abundance of potential miRNA targets suggest that miRNAs are likely to be involved in a broad spectrum of human diseases. In addition, components required for miRNA processing and/or function have also been implicated in fragile X mental retardation [135], DiGeorge syndrome [58–60] and cancer [136], pointing at a general involvement of miRNAs in disease.

### 5.1. miRNAs and cancer

More than half of the human miRNA genes are located at sites known to be involved in cancers, such as fragile sites, minimal regions of loss of heterozygosity, minimal regions of amplification, or common breakpoint regions. Such locations suggest that some miRNAs are involved in tumorigenesis [43]. miRNA expression profiles of a large number of human tumor samples show that miRNAs are sometimes upregulated [43], but generally downregulated in tumors [131]. Furthermore, nearly all miRNAs are differentially expressed in different cancers. Since the miRNA profiles reflect the developmental lineage and differentiation state of tumors, these profiles can be used to classify poorly differentiated tumors [131]. The roles for some miRNAs have been investigated in more detail (Table 1).

The cluster of the *mir-15* and *mir-16* genes lies in a region that is deleted in more than half of B-cell chronic lymphocyte leukemias and expression of miR-15 and miR-16 is downregulated in the majority of these leukemias [137]. The *mir-143* and *mir-145* genes also reside in a genomic cluster. Expression of both miRNAs is downregulated in colon cancer tissue as well as in several cell lines derived from other types of cancers [138]. The *mir-155* gene lies in the non-coding *BIC* RNA transcript. Both the expression of *BIC* RNA and miR-155 are upregulated in several types of lymphomas, especially in diffuse large B-cell lymphomas [139,140]. Patients with activated B-cell lymphomas have worse prognosis than patients with germinal center lymphomas, which is reflected by the absolute levels of miR-155 in these cancers [140]. The human *let-7* miRNA is downregulated in several lung cancers and is able to inhibit growth of lung cancer cells in vitro [111,141]. In addition, *let-7* levels are lowest in patients with the lowest postoperative survival; thus, *let-7* is also a good indicator for the prognosis of these types of cancers. The *mir-17-92* cluster is located in a region that is often amplified in human B-cell lymphomas and miRNA levels from this cluster are increased in B-cell lymphomas. Overexpression of the *mir-17-92* cluster prevents apoptosis and accelerates the formation of c-Myc-induced B-cell lymphomas in mouse models [142]. Interestingly, c-Myc itself also upregulates the expression of miRNAs from the *mir-17-92* cluster and the cell cycle promoter E2F1, a target for some of these miRNAs, resulting in a tightly controlled proliferative signal [143].

## 5.2. miRNAs and viruses

miRNAs have been identified in several mammalian viruses, including many members of the herpesvirus family [32], such as Epstein-Barr virus [33] and Kaposi sarcoma-associated virus [144], simian virus 40 [145] and possibly the human immunodeficiency virus [146]. For most of these miRNAs the functions are unknown, but they are expected to regulate the expression of viral and host genes for their survival and propagation in infected cells. For example, two miRNAs encoded by simian virus 40 target early viral mRNAs for cleavage. This reduces the expression of the viral T antigens, which makes the infected cells less susceptible to cytotoxic T cells and thus enhances the probability of successful infection [145]. Recently it has also been shown that endogenous miRNAs can mediate antiviral defense. The endogenous miR-32 prevents the accumulation of the retrovirus primate foamy virus type 1 (PFV-1) in human cells. However, a PFV-1-encoded protein, Tas, is able to partially suppress the miRNA-induced inhibition of PFV-1 [97]. Together, this indicates that the miRNA machinery is involved various aspects of human viral infections.

## 6. Conclusions

Since the discovery of *lin-4* and *let-7* it has become apparent that miRNAs form an important and abundant class of post-transcriptional gene regulators, which are widely present in multicellular organisms, ranging from plants to humans. Animals encode hundreds of miRNAs, of which the vast majority have unknown functions. Nevertheless, the limited set of characterized miRNAs indicates that miRNAs can act in diverse biological processes. In addition, mice and zebrafish which are defective in miRNA production or function show that miRNAs are essential for vertebrate development, and are likely to be involved in differentiation and/or maintenance of tissue and cell identity. The current set of miRNAs is predicted to regulate several thousands of target mRNAs, which may go up to 30% of all protein-coding genes [10,11,101]. This number might still increase because many additional miRNAs are predicted, even up to a thousand in vertebrate genomes [9]. The verification of the existence of these miRNAs and their interaction with target genes will be the key to find the function of all individual miRNAs during development, disease and other cellular processes.

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