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The Diverse Functions of MicroRNAs in Animal Development and Disease

Review

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MicroRNAs (miRNAs) control gene expression by translational inhibition and destabilization of mRNAs. While hundreds of miRNAs have been found, only a few have been studied in detail. miRNAs have been implicated in tissue morphogenesis, cellular processes like apoptosis, and major signaling pathways. Emerging evidence suggests a direct link between miRNAs and disease, and miRNA expression signatures are associated with various types of cancer. In addition, the gain and loss of miRNA target sites appears to be causal to some genetic disorders. Here, we discuss the current literature on the role of miRNAs in animal development and disease.

Introduction

Four types of silencing-related small RNAs have been found in animals over the past few years: small interfering RNAs (siRNAs), microRNAs (miRNAs), and, more recently, repeat associated small interfering RNAs (rasiRNAs) and Piwi-interacting RNAs (piRNAs).

siRNAs are usually derived from double-stranded RNA, which originates from viruses or overlapping transcripts. Generally, siRNAs trigger mRNA degradation by binding to their targets with perfect complementarity (reviewed by Meister and Tuschl, 2004).

Genomic repeats and retrotransposons give rise to rasiRNAs, and these have been found in *Drosophila* (Vagin et al., 2006; Saito et al., 2006). Interestingly, rasiRNAs arise primarily from the antisense strand of genomic repeats, and they seem to function through a distinct small RNA pathway involving Piwi proteins in flies.

A germline-specific class of vertebrate small RNAs is formed by piRNAs, which are 29–30 nt-long RNAs that act by binding to Piwi proteins. piRNAs are not associated with repeats; however, the encoding genes are localized in clusters in the genome, and piRNAs within a cluster map to only one strand of the genomic DNA (reviewed by Kim, 2006).

miRNAs are currently well established as a class of ~22 nt endogenous, noncoding small RNAs that influence mRNA stability and translation. Although the first miRNA, *lin-4*, was found in 1993 (Lee et al., 1993; Wightman et al., 1993), it took until 2000 before the miRNA field took off after the discovery of the highly conserved *let-7* small RNA (Reinhart et al., 2000).

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Since then, several groups have applied small-RNA-cloning strategies to identify new small RNAs in vertebrates and invertebrates (Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2001). More recently, hundreds of miRNAs have been found in many animal species by computational predictions, experimental approaches, or combined strategies (reviewed by Berezikov et al., 2006).

miRNA genes are transcribed by RNA polymerase II as capped and polyadenylated primary miRNA transcripts (pri-miRNA) (Cai et al., 2004; Lee et al., 2004). The RNase III enzyme Drosha initiates the nuclear processing of the pri-miRNA into an ~70 nt precursor miRNA (pre-miRNA) (Lee et al., 2003) (Figure 1). The double-stranded RNA binding protein DGCR8 interacts with Drosha to form the microprocessor complex (reviewed by Kim, 2005). Pre-miRNAs are exported from the nucleus by binding to the nucleocytoplasmic transport factor Exportin-5 (reviewed by Kim, 2004). Maturation of the pre-miRNA into an imperfect RNA duplex, with 2 nt 3′ overhangs, is mediated by the cytoplasmic enzyme Dicer (reviewed by Hammond, 2005). The strand of the duplex with the weakest base pairing at its 5′ terminus is preferably loaded into the RNA-induced silencing complex (RISC) (reviewed by Hutvagner, 2005).

The miRNA guides the RISC complex to the 3′UTR of target mRNAs. Animal miRNAs usually base pair with imperfect complementarity to their target. The seed region (nucleotides 2–8) of miRNAs is most important for target recognition and silencing (Lewis et al., 2005; Doench and Sharp, 2004). Association of miRNAs with their target mRNAs inhibits translation, but data on the exact mechanism of translational repression remain unclear due to the fact that both the initiation and elongation steps of translation are thought to be affected (Pillai et al., 2005; Petersen et al., 2006; reviewed by Valencia-Sanchez et al., 2006). Repressed mRNAs are present in cytoplasmic foci called P-bodies, which are known sites of mRNA destabilization (reviewed by Bruno and Wilkinson, 2006). Recent data suggest that, indeed, miRNA-mediated repression might have a profound impact on target mRNA levels, possibly via de-adenylation of the mRNA (Bagga et al., 2005; Lim et al., 2005; Giraldez et al., 2006; Valencia-Sanchez et al., 2006).

Recent advances in analyzing the spatial expression of miRNAs have shown that miRNAs are expressed in a very tissue-specific manner during development (Wienholds et al., 2005; Kloosterman et al., 2006; Ason et al., 2006; Aboobaker et al., 2005). This indicated that miRNAs are possibly involved in specifying and maintaining tissue identity. The conservation in expression of *miR-1*, for example, in muscles; *miR-124* in the central nervous system; and *miR-10* in anterior-posterior patterning suggests that the functions of these and possibly many other miRNAs have been conserved (also see Figure 2).

Functions of miRNAs

Loss of All miRNAs

From their conservation and expression patterns, it is clear that miRNAs might play important roles in

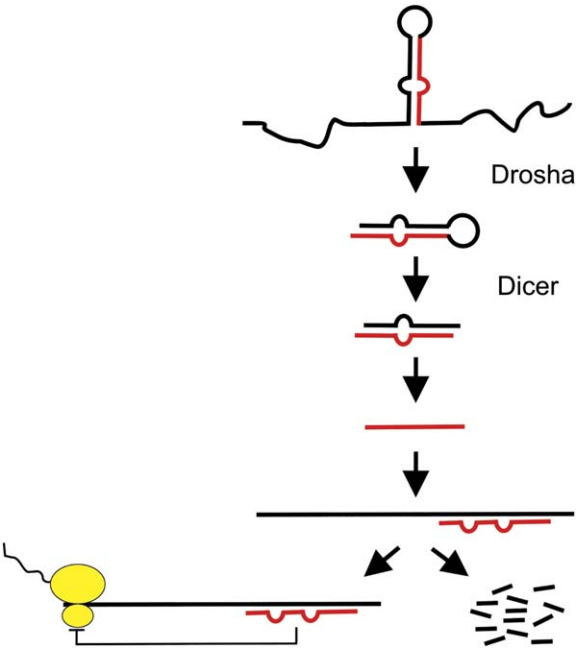

Figure 1. Schematic Overview of miRNA Biogenesis and Mechanism of Action

development, and, indeed, animals without miRNAs cannot live or reproduce (Bernstein et al., 2003; Ketting et al., 2001; Wienholds et al., 2003). Mice deficient in *Dicer* die at embryonic day 7.5 and lack multipotent stem cells (Bernstein et al., 2003). In addition, conditional inactivation of *Dicer* in embryonic stem cell lines compromises proliferation and differentiation; however, these effects might also be attributed to changes in centromeric silencing rather than to compromised miRNA production (Kanellopoulou et al., 2005; Murchison et al., 2005). Also, *Drosophila* mutants lacking *Dicer-1* in the germline stem cells show that the miRNA pathway is essential for stem cell division and to bypass the G1/S checkpoint of the cell cycle (Hatfield et al., 2005). However in zebrafish, *Dicer*-deficient primordial germ cells were transferred to wild-type embryos, and these could grow into fertile zebrafish including a germline that lacks *Dicer* and miRNAs (Giraldez et al., 2005). Together, these data show that not all organisms employ the same mechanisms to proliferate and differentiate pluripotent stem cells, and that, at least in zebrafish, Dicer and miRNAs are not required for germline stem cell development. Although the zebrafish zygotic *Dicer* mutant lives for almost 2 weeks due to maternally contributed Dicer (Wienholds et al., 2003), analysis of the maternal zygotic *Dicer* mutant embryos showed that zebrafish can differentiate multiple cell types in early development without miRNAs, but that morphogenetic processes are severely affected (Giraldez et al., 2005). Several conditional approaches to knock out *Dicer* in the mouse have been taken to circumvent the embryonic lethality of *Dicer* null mutants. These studies have indicated that Dicer, and probably also miRNAs, is essential for morphogenesis of the skin (Andl et al., 2006; Yi et al., 2006), lung epithelium (Harris et al., 2006), and the vertebrate limb (Harfe et al., 2005).

miRNAs as Regulators of Developmental Timing

The first miRNA, *lin-4*, was discovered in *C. elegans* as a small RNA species with antisense complementarity to the heterochronic gene *lin-14* (Lee et al., 1993; Wightman et al., 1993). Negative posttranscriptional regulation of *lin-14* by *lin-4* is essential for the formation of a temporal gradient of the LIN-14 protein, ensuring proper transition between *C. elegans* larval stages. Besides *lin-14*, the heterochronic gene *lin-28* is also a target of *lin-4. lin-28* homologs also exist in animals, including mouse and human. The *lin-28* 3′UTR harbors target sites for *miR-125a* and *let-7b* (homologs of *C. elegans lin-4* and *let-7*) and is expressed and downregulated during development (Moss and Tang, 2003).

The *let-7* miRNA regulates heterochronic genes in a similar manner as *lin-4*, and the complementary elements conferring regulation by *let-7* are in some cases evolutionarily conserved, e.g., *lin-41* (Pasquinelli et al., 2000). Together with the finding that *let-7* regulates the *C. elegans* hunchback homolog *hbl-1* and several other transcription factors during the larval-to-adult transition, this indicates that *let-7* acts as a master switch controlling temporal patterning (Abrahante et al., 2003; Grosshans et al., 2005; Lin et al., 2003). However, the *let-7* family miRNAs *miR-48*, *miR-84*, and *miR-241* also play a role in the *C. elegans* heterochronic pathway to control cell fate transitions (Li et al., 2005).

miRNAs Involved in Signaling Pathways

Although a role in developmental timing appeared to be the critical function for the *lin-4* miRNA, recently a role was proposed for *lin-4* in regulating life span, possibly through the insulin/insulin-like growth factor-1 pathway (Boehm and Slack, 2005). Overexpression of *lin-4* resulted in a prolonged life span, and *lin-4* loss-of-function mutations caused premature death. These data suggest a dual function for *lin-4* in controlling developmental timing during embryonic development and affecting life span through a major signaling pathway.

The Notch signaling pathway is essential for patterning and development, and studies in *Drosophila* have shown that Notch-targeted genes are regulated by

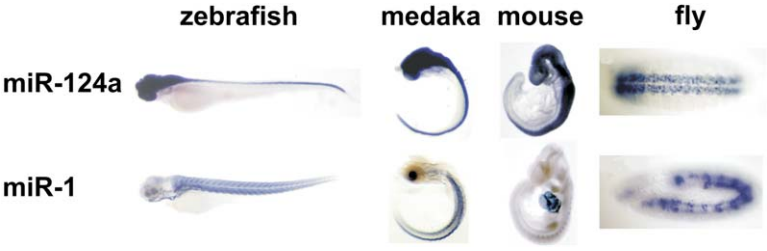

Figure 2. Expression of *miR-124a* and *miR-1* in Zebrafish, Medaka, Mouse, and Fly

The expression of *miR-124a* is restricted to the brain and the spinal cord in fish and mouse or to the ventral nerve cord in the fly. The expression of *miR-1* is restricted to the muscles and the heart in the mouse. The conserved sequence and expression of *miR-1* and *miR-124a* suggests ancient roles in muscle and brain development. The fly and medaka pictures were generously provided by Eric Lai and Brandon Ason, respectively.

miRNAs via conserved motifs (Lai et al., 2005). Ectopic expression of some of these miRNAs induces phenotypes that are reminiscent of Notch pathway loss of function. This repression of Notch-induced transcripts might be necessary to prevent their overexpression.

Several positive and negative-feedback loops exist that incorporate miRNAs. The *C. elegans* miRNA *miR-61* is a direct transcriptional target of LIN-12/Notch and functions in a positive-feedback loop promoting a secondary vulval cell fate, thus suggesting that *miR-61* plays a major role in specifying cell fate (Yoo and Greenwald, 2005).

Photoreceptor differentiation in the *Drosophila* eye is mediated by *miR-7*. This miRNA functions in a reciprocal negative-feedback loop to inhibit the expression of the YAN protein, which acts as a transcriptional repressor of *miR-7*. The expression of *miR-7* is initially triggered by EGF signaling, which results in phosphorylation and inactivation of YAN, generating a stable change in gene expression (Li and Carthew, 2005). Thus, major signaling pathways may induce changes in gene expression in the first place, but may use miRNAs to stabilize the resulting expression program. It is still unclear through which target genes outside the feedback loop *miR-7* promotes photoreceptor differentiation.

Although not directly related to signaling, miRNAs more often reside in feedback loops to control their own expression. Granulocytic differentiation is enhanced by *miR-223* expression (Fazi et al., 2005). Before the differentiation process, the transcription factor NFI-A allows weak expression of *miR-223*. Upon stimulation with retinoic acid, NFI-A is replaced by the transcription factor C/EBPα, which induces high expression of *miR-223*, which, in turn, represses the expression of NFI-A post-transcriptionally in a manner similar to *miR-7* and YAN.

The expression of *miR-7* in flies and *miR-61* in worms is induced by EGF and Notch signaling, respectively. However, miRNAs might also function upstream of signaling pathways. Hoxb8 is a transcription factor that mediates retinoic acid-induced expression of Sonic hedgehog, a signaling molecule that regulates anterioposterior patterning in limb buds. *miR-196* functions as a secondary regulator of *hoxb8* expression in the vertebrate hindlimb, where the primary level of *hoxb8* regulation appears to be transcriptional (Hornstein et al., 2005).

miRNAs in Apoptosis and Metabolism

Forward genetic screens in flies have led to the discovery of miRNAs involved in programmed cell death. The *bantam* miRNA accelerates proliferation and prevents apoptosis by regulating the proapoptotic gene *hid* (Brennecke et al., 2003). Similarly, *miR-14* functions as a cell death suppressor, although its cellular target is unknown (Xu et al., 2003). However, fly *miR-14* mutants display another phenotype: they are obese and have elevated levels of triacylglycerol. This phenotype shows that *miR-14* plays a role in fat metabolism. In a similar gain-of-function screen for genes affecting tissue growth, the *miR-278* locus was identified (Teleman et al., 2006). *miR-278* mutants are lean and have elevated insulin production, implicating this miRNA in energy homeostasis, possibly by interacting with the *expanded* transcript, which is known to be involved in growth control. In vertebrates, *miR-375* is expressed in the pancreatic island and suppresses glucose-induced insulin secretion (Poy et al., 2004). The *myotrophin* (*Mtpn*) gene was validated as a target of *miR-375* and siRNA-mediated knockdown of *Mtpn* mimicked the effect of *miR-375* on insulin secretion.

miRNAs Involved in Myogenesis and Cardiogenesis

The sequence of *miR-1* is conserved from worms to mammals. It is highly expressed in the muscles of flies and the muscles and heart of mice (Aboobaker et al., 2005; Zhao et al., 2005) (Figure 2). Interestingly, a genetic knockout of *Drosophila miR-1* does not affect the formation and physiological function of the larval musculature, but malformation of the muscles and death are only triggered upon feeding and growth (Sokol and Ambros, 2005). Thus, some miRNAs might not be essential for establishing a tissue type, but they are required for subsequent growth and maintenance of the tissue. In mouse, *miR-1* expression is directed by muscle-differentiation regulators, such as serum response factor, MyoD, and Mef2, in the same cells as one of its targets, Hand2 (Zhao et al., 2005). Although there is no genetic knockout of *miR-1* available, overexpression of *miR-1* results in developmental arrest, thin-walled ventricles, and heart failure due to premature differentiation and proliferation defects of myocytes.

In cell culture, *miR-1* promotes myogenesis by targeting histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle differentiation (Chen et al., 2006). HDAC4 is also a possible target of *miR-140* during osteoblast differentiation and skeletogenesis, showing that two different miRNAs can target the same gene during differentiation (Tuddenham et al., 2006). *miR-1* localizes in a genomic cluster with *miR-133*, but these two miRNAs differ in their seed sequence, and they have distinct functions (Chen et al., 2006). In contrast to *miR-1*, *miR-133* inhibits muscle differentiation and promotes proliferation by repressing serum response factor.

Although *miR-181* is hardly detectable in skeletal muscle, this miRNA is strongly upregulated during myoblast differentiation and inhibits the expression of HoxA11, which is a repressor of differentiation (Naguibneva et al., 2006). *miR-181* might be involved in establishing a muscle phenotype, while *miR-1* and *miR-133* are involved in muscle maintenance. In addition, *miR-181* can induce the differentiation of hematopoietic stem cells to B-lineage cells, demonstrating a dual role for a single miRNA in different cell types (Chen et al., 2004).

miRNAs in the Brain

The brain represents a complex tissue, with multiple different cell types. Many miRNAs are expressed in specific brain regions or neurons in vertebrates, suggesting their importance in brain functioning (Wienholds et al., 2005). Some miRNAs have been implicated in specifying asymmetric gene expression in chemosensory neurons in *C. elegans* (Chang et al., 2004; Johnston and Hobert, 2003). The *lsy-6* miRNA is expressed in left neurons, and *miR-273* is expressed in right neurons; this reciprocal expression is essential for neuronal asymmetry.

In the mammalian brain, *miR-134* was found to localize to synaptic sites in rat hippocampal neurons (Schratt et al., 2006). *miR-134* can inhibit the expression of the protein kinase Limk1, which controls the development of dendritic spines. The silencing can be released upon extracellular stimuli, resulting in spine growth.

Another miRNA, *miR-132*, is expressed in cortical neurons and is a target of the transcription factor

cAMP-response element binding protein (CREB) (Vo et al., 2005). Inhibition of *miR-132* attenuates neuronal outgrowth, and the effects on neuronal morphogenesis might be mediated by the GTPase-activating protein p250GAP.

Although these two examples propose roles for miRNAs in neuronal outgrowth and plasticity, *miR-124a* is involved in the differentiation of neural progenitors into mature neurons by degradation of nonneural transcripts (Conaco et al., 2006). *miR-124a*, together with *miR-9* and *miR-132*, is targeted by the transcriptional repressor REST in nonneural cells.

The *miR-430* family of miRNAs, which is the major miRNA family expressed during early zebrafish development, performs a more general role in zebrafish brain morphogenesis (Giraldez et al., 2005). In addition, this miRNA clears the embryo of maternal mRNAs to promote the maternal-to-zygote transition (Giraldez et al., 2006).

The Expression of miRNAs and Their Targets

The studies of *miR-7* and YAN in *Drosophila*, *miR-196* and Hoxb8a in mouse, and *miR-223* and NFI-A during granulopoiesis indicate that miRNAs and their targets might have reciprocal expression patterns, i.e., if the miRNA is expressed at a high level, the target is lowly expressed. This trend toward mutually exclusive expression of miRNAs and their targets has been shown on a larger scale by independent studies in mammals (Farh et al., 2005; Sood et al., 2006) and *Drosophila* (Stark et al., 2005). There are five important results from these studies. First, mRNAs are, in general, expressed at higher levels prior to miRNA expression. A very nice biological example of this is the clearance of maternal mRNAs by the *miR-430* family (Giraldez et al., 2006). Second, nonconserved target sites are preferentially found in genes that are not expressed in the tissue in which the miRNA is expressed. These might thus be mRNAs that randomly acquired target sites during evolution, which could persist because there is no selection against them. Third, conserved targets are expressed in the tissue in which the miRNA is expressed, but usually at lower levels compared to the surrounding tissue. Fourth, mRNAs that are preferentially coexpressed with an miRNA specifically avoid target sites for that miRNA, and these are referred to as antitargets. Antitargets could cooperate with miRNAs to establish the correct biological environment in a particular cell lineage or could be involved in basic processes common to all cells. Fifth, housekeeping genes have shorter UTRs than other messengers, and the target site density is lower compared to genes with longer 3′UTRs, probably to avoid targeting by miRNAs.

In principle, there are two ways that miRNAs could regulate their target (Bartel and Chen, 2004). Fine-tuning targets contain just one site for one miRNA and confer only weak regulation. This is the case for the majority of targets (Stark et al., 2005). In these instances, weak regulation of a target might become significant when the target is already expressed at very low levels in the cell. But why would tuning targets exist in the first place when, for many genes, there is not even an effect of the loss of a single allele (resulting in an expected 50% loss at the protein level)? Probably, these effects are not meaningful at the level of an individual, but might only become apparent from an evolutionary perspective, where small differences in gene expression affect the survival of individuals at the population level. The second class comprises switch targets, where multiple target sites are present in the 3′UTR resulting in strong repression. These could be target sites for just a single miRNA or target sites for different miRNAs, resulting in cooperative regulation provided that the miRNAs are expressed in the same cells. Switch targets could be rare, and the targets for many genetically identified miRNAs contain multiple sites, indicating that they are strongly regulated.

In light of the incidence of these two types of miRNA-mediated regulation, it does not come as a surprise that developmental defects caused by miRNA knockdown are limited (Leaman et al., 2005) and that so few miRNAs have been found in genetic screens. Even without the miRNA, the expression of its target mRNA seems to remain low in the tissue in which the miRNA is expressed compared to surrounding tissues (Farh et al., 2005). In addition, many miRNAs are part of families with related sequence that are potentially involved in the same pathways (Abbott et al., 2005). Thus, redundancy also plays a significant role in miRNA biology. Indeed, many miRNAs have multiple copies in the genome or reside in families of related sequence, and these family members are often coexpressed from a single transcript (e.g., *miR-182*, *miR-183*, and *miR-96* or *miR-221* and *miR-222*) (Wienholds et al., 2005). Interestingly, there are not many conserved miRNAs of unrelated sequence that are coexpressed in the same tissues (Wienholds et al., 2005). In those cases in which there are overlapping expression domains, it would be interesting to see if there is a higher than random cooccurrence of target sites for these miRNAs (e.g., *miR-1* and *miR-133* in muscles, *miR-7* and *miR-375* in the endocrine pancreas, or *miR-183* and *miR-200* in hair cells). On the other hand, from an evolutionary perspective it seems difficult to understand how several identical miRNAs could be maintained when they play an identical role during development. The expression of each of these copies could vary both in time and in space so that each copy adopts its own specific function and the pattern obtained with a probe for the mature miRNA provides a total picture. Alternatively, the many copies might be essential for establishing a high concentration of miRNAs inside the cell that meets the requirement of regulating hundreds of target genes. Also, the variation in the sequence of miRNA family members might help in fine tuning the expression of target genes. Members of the mixed pool of these related miRNAs could all recognize the same targets by seed pairing, but they could vary a little in the overall degree of complementarity, and, together, they could provide the required degree of regulation.

miRNAs Involved in Cancer

Altered miRNA Expression in Cancer

The initial evidence that miRNAs play a collaborating role in cancer came from a study on chronic lymphocytic leukemia (Calin et al., 2002). A region containing *miR-15* and *miR-16* at chromosome 13q14 is deleted in the majority of chronic lymphocytic leukemia cases. Follow up studies indicated that a common feature of human miRNAs is that they are located at genomic regions involved in cancer, such as common breakpoint regions

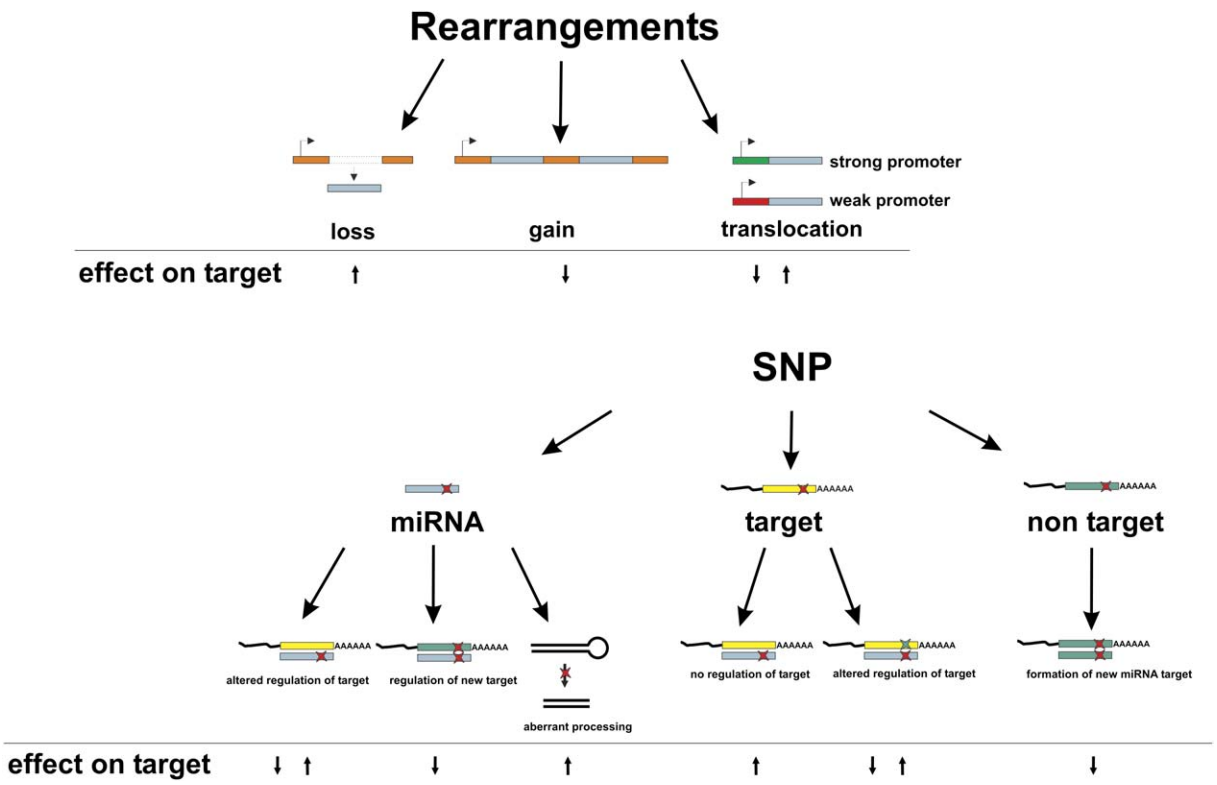

Figure 3. Mechanisms that Link miRNAs to Disease

There are two possible scenarios: (1) either the expression level of the miRNA changes due to genomic rearrangements; or (2) there is a gain or loss of an miRNA-target interaction due to a mutation in a 3′UTR or a mutation in the miRNA.

and fragile sites (Calin et al., 2004). In addition, among three cancer types, analysis of the DNA copy number of genomic regions containing miRNAs identified 26 miRNAs with a gain of gene copy number and 15 miRNAs with a loss (Zhang et al., 2006). It is obvious that miRNAs located in deleted or amplified regions in cancer samples have altered expression levels (Figure 3).

From several studies, it is now clear that differential miRNA expression is associated with tumor formation (Calin et al., 2004, 2005; Ciafre et al., 2005; Michael et al., 2003; Lu et al., 2005; Volinia et al., 2006; Yanaihara et al., 2006). A particularly interesting finding from a study by Lu et al. (2005) is that both the differentiation state and the developmental lineage of tumors are reflected in the miRNA profile (Lu et al., 2005). The analysis of parallel mRNA profiling did not lead to a similar clustering of tumor samples derived from the same lineage, suggesting that miRNA profiling of tumor samples might be more informative for cancer diagnosis compared to regular mRNA profiling. Indeed, the analysis of unique miRNA profiles of lung tumors revealed an important relationship between miRNA expression signatures and patient survival (Yanaihara et al., 2006). Also, the expression levels of, e.g., *miR-155* in B cell lymphoma patients and *let-7* in lung cancer are indicative for patient survival, showing that monitoring miRNA expression in cancer patients clearly has prognostic value.

Another striking finding presented in the study by Lu et al. (2005) is that most miRNAs are expressed at lower levels in tumors compared with normal tissues (Lu et al., 2005). Thus, a decrease in miRNA levels is associated with a loss of cellular differentiation. The widespread downregulation of miRNAs in cancers is possibly due to a failure at the Drosha-processing step (Thomson et al., 2006). These data support the suggested role of miRNAs in promoting differentiation and show that miRNAs can act analogous to tumor-suppressor genes.

Other studies show that some miRNAs are also upregulated in tumors versus normal tissues and thus act as oncogenes (Ota et al., 2004; Volinia et al., 2006). For example, the *miR-17-92* cluster is overexpressed in B cell lymphoma (Ota et al., 2004; He et al., 2005). However, this cluster comprises some of the few miRNAs expressed highly in embryonic stem cells, where expression is decreasing during embryonic development. In terms of miRNA biology, the *miR-17-92* cluster might be an exception in that it is not associated with differentiation, but rather specifies characteristics of stem cells, resembling the dedifferentiated state of malignancies.

Although no changes in the expression of genes acting as components of the miRNA machinery were observed in one study (Lu et al., 2005), some lung cancers were found to have reduced expression of *Dicer* (Karube et al., 2005), and ovarian tumors exhibited gains in DNA copy number of *Dicer* and *Argonaute 2* (Zhang et al., 2006), suggesting that indirect effects could also account for altered miRNA expression in tumors.

miRNAs Targeting Cancer-Related Genes

The studies described above clearly indicate that miRNAs are associated with cancer etiology, but this

does not provide evidence that altered miRNA expression is a cause or a consequence of tumor formation. In principle, miRNA downregulation could lead to increased expression of oncogenes, and miRNA upregulation could prevent the expression of tumor suppressors. When analyzing the miRNA expression profiles of solid tumors, Volinia and colleagues found that the predicted targets of 18 miRNAs that are dysregulated in tumors contain a significant overrepresentation of cancer-related genes (Volinia et al., 2006). For example, TGF-β-2 receptor is expressed in the epithelium of breast cancer and can be regulated by *miR-20a*, which is downregulated in breast cancer. Several other examples of miRNAs regulating cancer genes exist. *miR-15* and *miR-16* are downregulated in chronic lymphocytic leukemia. Both miRNAs target the antiapoptotic B cell lymphoma 2 (Bcl2) protein and can induce apoptosis by downregulating Bcl2 when expressed in cell lines (Cimmino et al., 2005). The *let-7* miRNA is expressed at low levels in human lung cancers (Takamizawa et al., 2004), and this phenomenon is strongly associated with a reduced expression of *Dicer* in lung cancer (Karube et al., 2005). Furthermore, expression of human *RAS* is regulated by *let-7* through multiple complementary sites in its 3′UTR, and the expression of *let-7* and *RAS* is inversely correlated in lung tumors, suggesting that *let-7* may act as a tumor suppressor (Johnson et al., 2005).

Apart from reduced miRNA expression in tumors, the overexpression of some miRNAs often promotes tumor formation. The *miR-17-92* cluster is amplified in hematopoietic malignancies (Ota et al., 2004). Overexpression of this cluster can act together with c-Myc to accelerate tumor development in a mouse model for B cell lymphoma (He et al., 2005). In addition, induction of the *miR-17-92* cluster by c-Myc reduces expression of antiangiogenic proteins, resulting in enhanced tumor angiogenesis (Dews et al., 2006). How delicately tumor proliferation is controlled is demonstrated by the direct transcriptional activation of *E2F1* and the *miR-17-92* cluster by c-Myc, while *miR-20a* and *miR-17-5p*, in turn, negatively regulate *E2F1* (O'Donnell et al., 2005). With the increasing number of human miRNAs that have been discovered over the past few years, miRNA overexpression screens can be performed to identify novel miRNA functions. In a genetic screen, *miR-372* and *miR-373* were found to allow proliferation of primary human cells that express oncogenic RAS and active p53, possibly by inhibiting the tumor suppressor *LATS2* (Voorhoeve et al., 2006). Interestingly, expression of *miR-372* and *miR-373* seems to be specifically associated with germ cell tumors, as most somatic tumors do not express these miRNAs. Similar to the *miR-17-92* cluster, these miRNAs are highly expressed in ES cells, implying that their in vivo function is reestablished in germ cell tumors to induce transformation.

In conclusion, because most miRNAs are thought to play a role during differentiation and maintenance of tissue identity, it is expected that the undifferentiated state of malignant cells is often correlated with a decrease in the expression of miRNAs. However, some of the miRNAs that have a natural function in maintaining a stem cell fate can be highly expressed during the transformation.

Disease Associated with Changes in the Sequence of the miRNA or the miRNA Target Site

miRNA Polymorphisms

Is altered miRNA expression, as observed in tumors, the only mechanism that connects miRNAs to disease? Apart from changes in miRNA expression levels, the interaction between the miRNA and its target could be disturbed due to mutations in either the miRNA itself or in its target site (Figure 3) (Plasterk, 2006). A mutation that affects the activity of the miRNA should preferably be located in the seed sequence of the mature miRNA (Lewis et al., 2005). To date, no systematic analysis of polymorphisms in miRNAs among diseased subjects has been performed, although polymorphisms have been detected among a cohort of 96 people representing the general population in Japan (Iwai and Naraba, 2005). Some single nucleotide polymorphisms (SNPs) were detected in the loop of the pre-miRNA, and these should not influence processing (Zeng and Cullen, 2003).

One C-to-A transition in mature *miR-30c-2* was detected, but this variant was still processed, and 11 people with a heterozygous phenotype appeared healthy. In contrast, one of the miRNAs from a variant of Kaposi's sarcoma-associated herpesvirus (KSHV) contains an SNP in the precursor stem loop that results in aberrant processing and, therefore, reduced miRNA function (Gottwein et al., 2006).

Polymorphisms in miRNA Target Sites

Gene expression is commonly regulated by regulatory motifs in the 3′UTR of mRNAs. In a systematic comparison of 3′UTR regions of protein-encoding genes across the mouse, human, rat, and dog genomes, 106 highly conserved and frequently occurring motifs were found (Xie et al., 2005). Strikingly, the length distribution of these motifs gave a peak at a length of 8 bases and had a strong tendency to end with an A, reminiscent of miRNA target sites. About 43.5% of a set of 207 human miRNAs matched with these conserved 8-mer motifs. The remaining miRNAs that do not contain complementary matches to any of the conserved motifs are evolving much more rapidly and may bind relatively few targets. Roughly one-half of the conserved motifs found in 3′UTRs appear to be related to miRNAs, and 5000 human genes (20% of the genome) contain conserved motifs, indicating the importance of miRNA-mediated gene regulation. In another study, miRNA targets were predicted by identifying mRNAs with conserved complementarity to the seed of the miRNA in four mammalian genomes (Lewis et al., 2005). A similar number of 5300 human genes were predicted as miRNA targets. The strong conservation of many miRNA target sites indicates that their regulation is important for cellular processes. Consequently, natural variation in miRNA target sites could result in aberrant regulation of gene expression, causing genetic diseases. Alternatively, new miRNA target sites could arise from mutations in 3′UTR sequences.

Variation in a Target Site for miR-189

Tourette's syndrome (TS) is a genetically influenced neuropsychiatric disorder that affects as many as 1 in 100 individuals (Abelson et al., 2005). In a search for candidate genes associated with TS, a patient was identified with a de novo chromosome 13 inversion. Three genes were found within the two breakpoints of the rearrangement, and *Slit and Trk-like family member 1*

(*SLITRK1*) was considered as the most likely candidate for further study because of its expression in brain regions implicated in TS. Subsequently, the *SLITRK1* gene was screened for mutations in 174 affected individuals. Besides a 1 base deletion in the coding region leading to a frameshift and a truncated protein, two identical occurrences of a sequence variation that mapped to a highly conserved nucleotide in the 3′UTR of the transcript were found. Because the two variations were found in distinct haplotypes, they probably arose independently. The polymorphism was located within the predicted binding site for *miR-189*, replacing a G:U wobble base pair with an A:U pairing. In the wild-type situation, the *SLITRK1* 3′UTR is regulated by *miR-189*, but the regulation is stronger in the variant with the polymorphism in the target site. In situ hybridization for *miR-189* and *SLITRK1* indicated developmentally regulated and overlapping expression patterns in neuroanatomical circuits most commonly implicated in TS. Although the mutation in the target site for *miR-189* is just one of the many rare mutations associated with this complex genetic disorder, it indicates that miRNAs might also be involved in the pathways that underlie disease.

Gain of a Target Site for miR-1 *and* miR-206

In a search for the genetic factors underlying the exceptional muscularity of Texel sheep, a quantitative trait locus (QTL) was identified with a major effect on muscle mass (Clop et al., 2006). Fine mapping of this QTL resulted in an interval on chromosome 2 that contained the myostatin gene (*GDF8*). Because loss of myostatin in mice, cattle, and human causes muscle doubling, this gene seemed a likely candidate for the muscle hypertrophy in Texel sheep. Although no polymorphisms were detected in the myostatin open reading frame, 20 SNPs were found in a 10.5 kb region spanning the sheep myostatin gene. One of these was a G-to-A transition and appeared to be virtually Texel specific. The mutation creates a target site for *miR-1* and *miR-206*. Both miRNAs are known to be strongly expressed in muscle, which is also the primary site of myostatin expression. Examination of the serum levels of myostatin showed a significant reduction of the protein in Texel sheep compared to controls. In addition, luciferase reporter assays with both the Texel myostatin 3′UTR and control UTRs lacking the polymorphism showed a moderate, but clear, reduction in the signal upon expression of either *miR-1* or *miR-206*, demonstrating direct inhibition of the Texel myostatin gene expression via its newly acquired miRNA target site. These findings indicate that the gain of an miRNA target site might significantly affect phenotypic variation. When searching among SNPs that map to the 3′UTR of human genes, 2490 SNPs were found that create a potential miRNA target site, and 2597 SNPs were found that destroy a predicted target site. Of this latter set, 483 SNPs affect a potential target site that is perfectly conserved among 4 mammalian species. This indicates that changes in miRNA-target interactions form a potent source of phenotypic variation. Notably, apart from destroying or creating an miRNA target site, a mutation in either a target site or an miRNA can also modulate the regulation, i.e., the strength of the regulation may also depend on the degree of complementarity between the miRNA and the target, as has been shown for *SLITKR1* and *miR-189* (Abelson et al., 2005).

Several examples exist for polymorphisms in 3′UTRs that alter mRNA stability and predispose people to disease, e.g., Fang et al., 2005. Although in these cases it is unclear if changes in miRNA binding might be associated, the above-described examples indicate that polymophisms in *cis*-regulatory elements in 3′UTRs, such as miRNA target sites, might result in phenotypic variation and influence susceptibility to disease.

miRNAs Associated with Viral Infection

Apart from multicellular eukaryotes, many miRNAs have been found in viruses (Cullen, 2006; Pfeffer et al., 2004, 2005). The small size of miRNAs makes them especially attractive as effector molecules during viral infection. Experimental evidence for the existence of viral miRNAs has come from several studies that detected miRNAs in herpesviruses and polyoma viruses (Cai et al., 2005; Grey et al., 2005; Grundhoff et al., 2006; Samols et al., 2005; Pfeffer et al., 2004, 2005). How does a virus use miRNAs to aid infection? A tip of the iceberg is provided by the examination of miRNAs from the SV40 virus, one of which induces degradation of viral mRNA coding for T-antigens (Sullivan et al., 2005). Although this does not affect SV40 replication, it makes viral-infected cells less sensitive to cytotoxic T cells. Alternatively, viral miRNAs could regulate the expression of their host genes, but, to date, no cellular genes have been described that are targeted by viral miRNAs. On the contrary, cellular miRNAs could be evolved to modulate the replication of the virus. Indeed, the accumulation of a retrovirus was shown to be reduced in human cells by the activity of *miR-32*, indicating that the miRNA machinery is exploited for defense against viruses (Lecellier et al., 2005). Viruses, in turn, can use an miRNA to facilitate their replication, as is the case for *miR-122* (Jopling et al., 2005). This liver-specific miRNA directly interacts with the 5′ noncoding region of the viral genome, which is essential for viral RNA maintenance. These examples show that miRNAs did not only evolve to regulate gene expression programs during embryonic development, but also coevolved with viral invaders to restrict, or even enhance, their replication.

Concluding Remarks

The importance of miRNAs during animal development is clear from several points. First, animals cannot live without miRNAs. Second, there is a large repertoire of conserved miRNAs. Third, mRNAs contain conserved miRNA target sites. Fourth, most miRNAs are highly abundant and exhibit striking tissue-specific expression. In addition, the role of miRNAs in several disease-related processes is now well established. It does not require much courage to predict that the coming years will bring numerous examples of genetic disease caused by dysfunction of miRNAs.

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