[Organogenesis 3:1, 25-33, January/February/March 2007]; ©2007 Landes Bioscience

Review

miRNAs: Miracle or Mirage?

The Limes* Against the Barbaric Floods of Leaky and Undesired Transcripts

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Original manuscript submitted: 11/10/06
Manuscript accepted: 12/05/06

Previously published online as an *Organogenesis* E-publication:
http://www.landesbioscience.com/journals/organogenesis/abstract.php?id=3670

KEY WORDS

microRNA, Dicer, canalization, embryogenesis, cancer

ACKNOWLEDGEMENTS

I am very grateful for the support by Dr. Sarah E. Millar and for her helpful comments. Furthermore, I would like to thank Omar Harb, Candace M. Moore, Emily Chu and Peggy Myung for their comments and encouragement.

ABSTRACT

The evolution of complex animals such as insects and mammals is achieved with surprisingly few additions in protein coding genes. MicroRNAs (miRNAs), a class of noncoding RNAs, have emerged as important regulators of organogenesis in insects, fish and mammals. The microRNA repertoire of animals has expanded significantly during evolution especially in vertebrates, insects and nematodes, accompanying the appearance of complex body plans. MicroRNAs therefore have gained enormous interest in recent years. They are now regarded as key modulators of gene expression in many tissues during embryogenesis, in adult organisms and in disease processes. Therefore, these small RNA molecules have entered the center stage of molecular biology and are promising candidates not only for the regulation of key biological processes such as proliferation and apoptosis, but also for therapy of human diseases.

INTRODUCTION

The field of RNA research has experienced a renaissance with the discovery of RNA interference (RNAi). Recently, studies in this area have intensified with the identification of other small RNAs and the realization that microRNAs (miRNAs) are natural RNAi agents. This renewed interest has been further fueled by the choice of the Nobel prize committee to honor Andrew Fire and Craig Mello for their work on RNAi just eight years after their important publication.[1] With these findings, there are great expectations for a better understanding of human disease along with hopes of novel therapeutic approaches. This review is intended to give an overview of current ideas of how microRNAs are generated, function and influence development and human disease.

MicroRNA BIOGENESIS AND FUNCTION

miRNAs are indeed small RNAs with the mature active form being approximately 22 nucleotides long. MiRNAs are derived from much larger transcripts (known as pri-miRNA) that are likely generated by RNA polymerase II. A hallmark of these transcripts is their ability to form hairpin structures containing sections of double stranded RNA. miRNAs can be located within introns of protein coding or noncoding genes, and in exons between genes (Miranda database, release 1.0 2005: about 62% intergenic, 34% in introns and 4% in exons). miRNAs can also occur in clusters, resulting in polycistronic transcripts. The double-stranded RNA structures in the primary transcript are substrates for a protein complex that contains RNase activity.[2] The responsible RNase, Drosha, is active in the nucleus. Drosha works in conjunction with DGCR8 (DiGeorge Syndrome critical region gene 8). DGCR8 directs Drosha to the cleavage sites of the pri-microRNA (about 11 nucleotides away from the beginning of the stem structure[3]). The product of Drosha-mediated RNA cleavage is termed the pre-miRNA. Pri-miRNA cleavage appears to be tightly regulated in early embryogenesis in mammals, though probably not in *C. elegans*.[4] Many mammalian mature miRNAs are not expressed in early embryogenesis even though their primary transcripts are present. In fact, the regulation of miRNA biogenesis can likely occur at all processing steps (transcription, Drosha-mediated processing, exportation to cytoplasm, Dicer-mediated cleavage). Pre-miRNAs are actively transported out of the nucleus into the cytoplasm by exportin-5, where they become the target of another protein complex, containing the RNaseIII enzyme Dicer. Dicer

*The limes was the line of demarcation or border line of the Roman Empire in the 2nd century A.D. (see also www.limes-in-deutschland.de/limes_english.html).

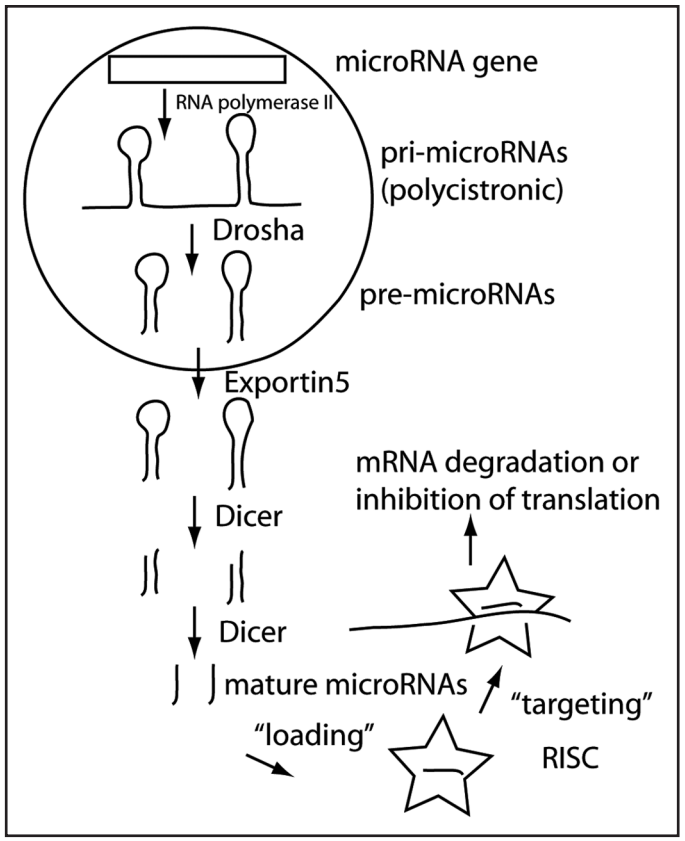

Figure 1. MicroRNA biogenesis. MiRNAs are mainly derived from RNA polymerase II transcribed genes. The primary transcript (pri-microRNA) is often polycistronic, containing several miRNas. In the nucleus, Drosha in conjunction with DRCG8 processes the pri-microRNAs into pre-microRNAs that are exported to the cytoplasm by Exportin5. In the cytoplasm, the mature miRNA is formed by the enzymatic activity of Dicer. The mature miRNAs is incorporated or "loaded" into the RISC (star shaped icon) that mediates miRNA-directed targeting of specific mRNAs.

produces the final product, the mature miRNA, which is loaded into the RNA-induced silencing complex (RISC[5]). MiRNA-loaded RISCs target mRNAs that contain at least partially complementary sequences to the miRNA (Fig. 1). Several methods have been applied to calculate the number of miRNA targets in different species. Target prediction algorithms integrate sequence complementary data, free energy values of the miRNA-mRNA complex, conservation of the target site between different species and the importance of the 5' seed sequence (nucleotides 2 to 7 of the mature miRNA) for binding the target mRNA. However, these different approaches predict somewhat different sets of target genes. Most estimates suggest that about one third of all human genes could be targets for microRNA inhibition[6] and that each microRNA family may target on average approximately 200 transcripts.[7] The reliability of these predictions is still controversial.[8] The latest astonishing and probably the most elaborate predictions go far beyond previous estimates, suggesting that there may be more than 25000 miRNAs in humans targeting more than 20000 mRNAs.[9]

The fate of the mRNA targeted by microRNAs remains less clear. Generally, these RISC-mRNA complexes end up in cellular substructures called P-bodies[10] where the mRNA is either stored for later release under certain conditions, i.e., stress, or degraded. miRNAs exert post-transcriptional repression via two main mechanisms: degradation of the targeted mRNA or its storage in P-bodies. Deadenylation of the targeted mRNA is associated with its appearance in P-bodies. In any case, the mRNA is prevented from undergoing translation.

MicroRNAs AND THE EVOLUTION OF THE 3'UTR IN EUMETAZOA

MicroRNAs have existed in animals for probably 600 million years and are present in almost all eumetazoa with the exception of the phylum porifera, which seems to be devoid of this class of small RNAs. The core set of eumetazoan microRNAs includes *miR-10* and *miR-100* (for nomenclature of miRNAs see Griffiths-Jones et al., ref. 11), which can be found in a variety of organisms from cnidaria to humans. There are another 18 ancient microRNAs that are shared by all deuterostomes and protostomes. From this point, there appears to have been a rapid enlargement of the microRNA pool during the evolution of several lineages, most prominently in vertebrates and again in eutherian mammals. There are even sets of microRNAs that appear to be entirely specific to humans.

The analysis of microRNAs can therefore be a powerful tool in phylogenetics. In this vein, miRNAs may also be helpful in clarifying the classification of certain animal groups. For example, planaria appear to be protostomes rather than deuterostomes (the two major subgroups of bilaterian animals) when analyzed by their microRNA repertoire. Many of the protostome-specific microRNA families, but not deuterostome-specific ones, have been found in planaria.[12,13]

There appears to be a strong correlation between the evolutionary appearance of new body plans and microRNA evolution. Because the target sequences and the sequences of the mature microRNAs are so small, new microRNAs may form de novo very easily and potentially contribute to the regulation of the expression patterns of a large set of genes. Consistent with this, there are indications that microRNAs have had a substantial impact on the evolution of the 3'UTR region of many genes. Large sets of genes seem either lack large 3'UTRs and/or microRNA target sites in their 3'UTR. These genes have been called anti-targets. However, for mRNAs that have target sites in their 3'UTR, these sites are frequently conserved between species indicating their biological significance. During evolution 3'UTRs have been surprisingly well conserved,[14] particularly for transcription factor genes in vertebrates. This supports the observation made in *Drosophila* that microRNAs can drive the evolution of 3'UTRs of target genes.[15]

Interestingly, the average size of 3'UTRs has increased during evolution,[16] concomitant with the expansion of the microRNA repertoire. In contrast, 5'UTR lengths is similar in fungi, plants, invertebrates and mammals.

microRNA FUNCTION: CONFERRING ROBUSTNESS TO DEVELOPMENTAL PROGRAMS?

Intriguingly, miRNAs and their targets are often not coexpressed, but rather have inverse expression patterns, with microRNAs being expressed in tissue domains adjacent to cells that express target mRNAs. Analysis of microRNA and target gene expression in *Drosophila* supports the idea that microRNAs have evolved to confer robustness to the gene expression pattern established by transcription factors. In this view microRNAs function as suppressors of leaky unwanted transcripts and as inhibitors of severe undesirable fluctuations of gene expression. Leaky and unwanted transcripts may produce ambiguous signals, potentially perturbing execution of the developmental program. The more complex the developmental program

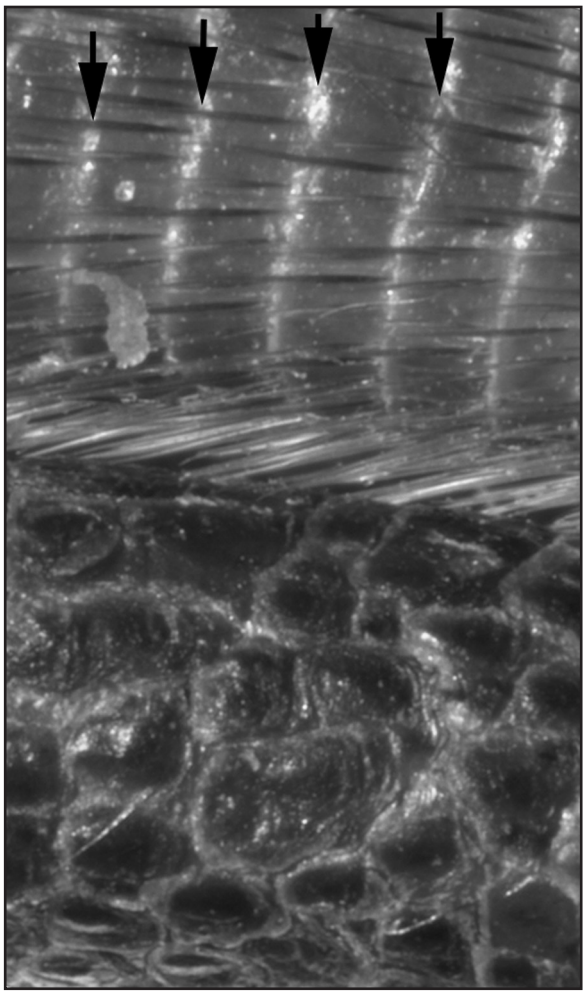

Figure 2. Disruption of regular scale pattern on tails of mice with epidermal specific loss of *Dicer*. Arrows indicate the regular lines separating scales on the tail of a control mouse (upper tail with hair). However, in mice with loss of *Dicer* function in the epidermis this regular pattern is destroyed (lower, darker and hairless tail).

becomes, the greater the potential contribution of microRNAs to its fidelity. However, Farh et al.[17] have shown that microRNAs can also function during development by dampening the expression of target genes that are coexpressed with the microRNA (see also *miR-278* in fat bodies of *Drosophila,* ref. 18). This mechanism may be important during differentiation and other processes requiring broad changes in expression that have to be accomplished quickly and with precision. Once we have a better picture of the total number of microRNAs in different phyla and their functions, we will be better able to address the question of whether and how microRNAs have contributed to the evolution of the different animal phyla, the diversification in certain animal groups (such as vertebrates and mammals) and whether the broadening of the influence of microRNAs on gene expression can influence the evolution of new body plans.

ERECTING A LIMES AGAINST UNWANTED mRNA TRANSCRIPTS: microRNA LEGIONS SECURING THE OUTCOME OF DEVELOPMENTAL PROGRAMS

The hypothesis that microRNAs may play an essential role in the process called canalization may help to explain one of the roles of microRNAs in animals. Canalization refers to a biological process conferring robustness and precision to gene expression patterns, a mechanism to "even out" deviations from the desired phenotype (reviewed by Hornstein and Shomron, ref. 19). MicroRNAs seem excellent tools for the execution of canalization. This hypothesis is based on several observations. One is that the phenotype of loss of microRNA function is far less dramatic than expected. For example, zebrafish development without the crucial mature microRNA-generating enzyme Dicer, and therefore without microRNA control over potentially thousands of genes, is surprisingly normal.[20] All the major tissues, organs and cell lineages seem to form in Dicer mutant fish. However, tissue morphogenesis proceeds abnormally, leading to a deformed embryo. The authors conclude from their analysis that patterning of fish embryos in the absence of Dicer occurs properly but is followed by irregular morphogenesis in a variety of tissues. This is also in concordance with microRNA expression patterns in zebrafish.[21] Most mature microRNAs are hardly detectable in early development and are strongly expressed at the end of organogenesis. This appears also to be true for mouse embryogenesis.[22]

This finding is in striking contrast to the restrictive model of messenger RNA gene expression during embryogenesis.[23] Messenger RNA expression patterns become less complex during development, and this restriction is believed to go along or be responsible for the restricted potential of specialized, differentiated somatic cells and tissues. In contrast, microRNA expression patterns become more complex during mouse, human and zebrafish development, and each tissue seems to acquire a very distinct microRNA expression signature. This in turn may actually enhance the already restrictive mRNA expression pattern during development by further limiting mRNA expression domains. This can be interpreted as supporting evidence for a crucial function of microRNAs in the process of canalization. In our own studies, we have observed a phenomenon that represents the loss of canalization: lack of Dicer expression leads to the disruption of the regular scale pattern on the tail the Dicer mutant mice (see Fig. 3).

Examples of microRNA Function: *miR-1* in Heart and Muscle. One of the few miRNAs that has been well studied in vertebrates and flies, *miR-1*, has a very specific mesodermal and, consequently, muscle- and heart-specific expression pattern (reviewed by Nguyen and Frasch, ref. 24). As such, *miR-1* may be a good candidate for mediating the muscle and heart phenotypes in zebrafish *Dicer* mutants. *miR-1* gene expression can be induced by muscle-associated transcription factors SRF, MyoD, and Myogenin[25] (Twist and Mef2[26] in myogenesis of *Drosophila*), and miR-1 can target the 3'UTR of HDAC4, a potent transcriptional repressor of muscle gene expression.[27] This initiates or maintains the transition from myoblast to myotubes. Therefore, *miR-1* plays an important role in myoblast differentiation.

Loss of function mutations of *miR-1* have been studied in Drosophila; however, the results are somewhat ambiguous. Two groups have generated miR-1 mutant flies and both show that the majority of the mutants die at a similar stage.[26,28] However, Kwon et al.[28] describe a much more severe phenotype in a substantial subset of embryos. These embryos show an expansion of cardiac progenitor cell populations and the loss of differentiation into cardiac and muscle cells. *miR-1* may have several functions in mesodermal cells during muscle differentiation: Suppression of unwanted transcripts, regulation of Notch/Delta signaling in early stages of cardiac and muscle cell differentiation, and regulation of muscle growth during larval stages. Altogether the results of Kwon et al.[28] complement

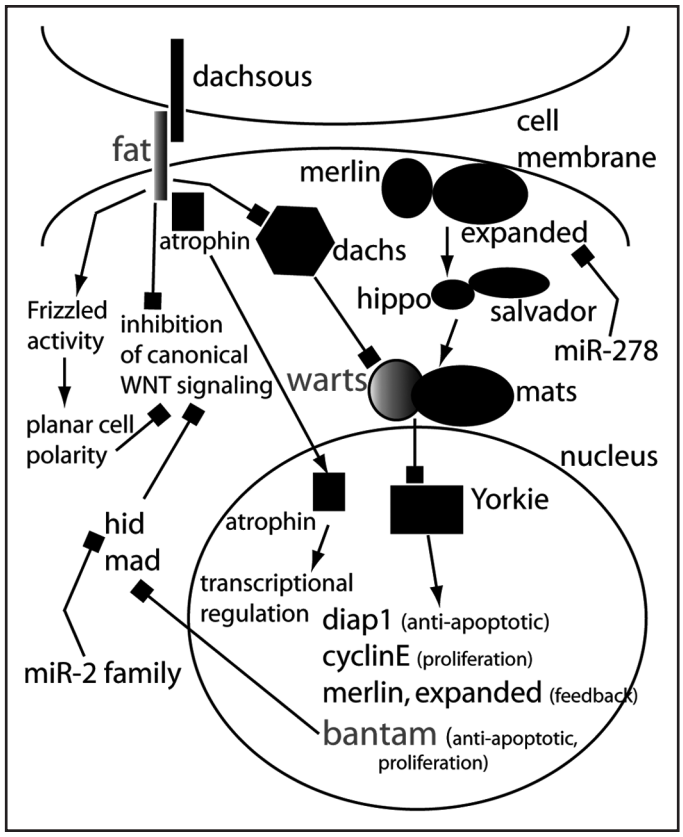

Figure 3. Model for the role of microRNAs in the regulation of 'tumor suppressor" pathways in *Drosophila*. The miRNA *bantam* is a key target of the transcriptional regulator Yorkie. *Yorkie* is under tight control of the Hippo pathway that consists of *merlin* and *expanded* at the membrane integrating signals from an unknown transmembrane receptor. In certain cell types in *Drosophila*, expanded mRNA is regulated by *miR-278*. Warts and Mats transduce the signal from Merlin/Expanded and Hippo/Salvador. Warts and Mats are also elements of Fat/Dachsous signaling (another *Drosophila* tumor suppressor pathway) through Dachs. Fat also can interfere with canonical Wnt signaling and activate noncanonical Wnt signaling. This could theoretically occur via the inactivation of *Yorkie* and *Bantam*: one of *Bantam*'s target mRNAs is *Hid*, which has been shown to inhibit canonical Wnt signaling.

previous studies using antisense-mediated depletion of *miR-1* in *Drosophila*.

LOSS OF FUNCTION ANALYSES OF microRNAs IN *DROSOPHILA* REVEAL BROAD ROLES FOR THIS CLASS OF REGULATORS

In addition to *miR-1*, four other miRNA loss-of-function mutants in *Drosophila* have been analyzed. The *Bantam* miRNA controls apoptosis and proliferation in *Drosophila*, indicating that miRNAs can influence the development in animals, especially the determination of organ and body size. *Bantam* targets mRNAs of apoptosis-inducing genes and negative regulators of proliferation. One direct target appears to be *hid*, a pro-apoptotic gene.[29] *Bantam* expression itself is regulated by the transcriptional coactivator Yorkie (Yki), a *Drosophila* homologue of the vertebrate yes-associated protein, Yap.[30,31] *Bantam* is thus an integral component of the Hippo tumor suppressor pathway and may also contribute to other tumor suppressor pathways.[32,33] The main target of the Hippo pathway appears to be *Yki*. Activation of the pathway leads to inactivation of *Yki* and consequently the suppression of proliferation and activation of apoptosis. Yap has been shown to have oncogenic activity, at least in mammary cells.[34] *Yap* also appears a key element of the 11q22 amplicon found in several types of tumors.[35] However, there has been no connection made between *Yap* and microRNAs in vertebrates so far. Moreover, *Bantam*, a target of Yki in *Drosophila*, can only be found in protostomes. Although the Hippo pathway is well established in *Drosophila*, its existence and regulation by microRNAs in deuterostomes is uncertain (for an overview of the *Drosophila* Hippo pathway and its interactions with miRNAs see Fig. 2).

Similar to *bantam*, the loss of function of *miR-14* in *Drosophila* also seems to involve the disturbance of apoptosis control resulting in reduced viability of the organism.[36] However, the exact molecular mechanisms by which *miR-14* exerts its function remain unclear. Interestingly, several apoptotic genes contain miR-14 target sites and one of them, *Drice*, shows elevated expression in *miR-14* mutant flies.

MiR-278 is highly expressed in the fat body of flies. The loss of *miR-278* in *Drosophila* results in lean flies while gain-of-function of this microRNA causes tissue overgrowth.[18] The loss of *miR-278* is accompanied by elevated insulin signaling, insulin resistance of the fat body, and aberrant expression of the gene *expanded* in the fat body. Interestingly, *expanded* is a member of the *ezrin/radixin/moesin* family and is part of the Hippo signaling pathway. *miR-278* and *bantam* therefore seem to be involved in the regulation of the same pathway. While *bantam* is activated by Yorkie, *miR-278* seems to be able to regulate an upstream component of the pathway, i.e., *expanded*. However, it seems unlikely that bantam and *miR-278* are involved in Hippo signaling in the same cell since no reports mention overlapping expression patterns.

An example that demonstrates how the differential expression of a miRNA in neighboring cells directs the fate of the cells comes from *miR-9a* mutant flies. Li et al[37] generated *miR-9a* mutant and overexpressing flies. *MiR-9a* is associated with the development of the nervous system. The authors show that *miR-9a* is important in the peripheral nervous system as well in establishing the correct pattern of sensory organ precursors (SOPs). Without *miR-9a*, ectopic SOPs form and overexpression of *miR-9a* leads to the loss of SOPs. *MiR-9a* achieves this mainly by regulating the transcription factor senseless. High levels of senseless activate proneural genes and SOP fate while low levels suppress proneural genes. Differential expression of *miR-9a* in SOPs and adjacent cells is one of the supportive factors that help to define accurately which cell becomes a SOP. *MiR-9a* therefore can be regarded as an example of the "classical" function of miRNAs: fine-tuning gene expression and conferring robustness to the gene regulatory network (reviewed by Cohen et al., ref. 38).

DICER AS A TOOL TO STUDY microRNA FUNCTION ON A LARGER SCALE

Loss of *Dicer* is an alternative and important tool in studying microRNA function. There is most likely only one *Dicer* gene in zebrafish, mice and humans; thus, targeting this gene has been a productive way to elucidate microRNA function in vertebrate development. Although the loss of *Dicer* in zebrafish correlates with the idea that microRNAs mediate canalization, the findings that mouse embryos without *Dicer* do not survive beyond embryonic day E7.5 is harder to explain.[39] One possibility is that microRNA function is different in mice and fish during early embryogenesis and that microRNAs in mice have essential functions in stem cell maintenance.

Table 1 **Phenotypes of mice with a tissue-specific loss of Dicer**

Tissue	Cre	Phenotype	Literature
Lung	Shh–cre (lung epithelium)	Branching defect, Fgf10 dysregulation, apoptosis induction.	Harris et al.[45]
Limb	Prx1–cre (limb bud mesenchyme)	Apoptosis	Harfe et al.[44]
Skin	K14–cre (epidermis and other squamous epithelia and their adnexa)	Apoptosis, loss of hair follicle stem cells, disturbance of epithelial–mesenchymal interactions	Andl et al.[47]
Skin	K14–cre (epidermis and other squamous epithelia and their adnexa)	Apoptosis, disturbance of epithelial–mesenchymal interactions	Yi et al.[46]
Immune system	CD4–cre (T cells)	CD8+ T cells development blocked; CD4+ T cells show increased apoptosis upon stimulation	Muljo et al.[42]
Immune system	Lck–cre (early stages of T cell development)	thymocyte cell number reduced, few peripheral T cells	Cobb et al.[43]

An alternative explanation is that Dicer has functions in addition to generating mature microRNAs: In several organisms, including those lacking microRNAs, Dicer is required for heterochromatin formation and centromeric silencing using siRNAs. In mouse embryonic stem cells (ES cells) the same function of Dicer has been demonstrated.[40] Furthermore, *Dicer* mutant mouse ES cells cannot differentiate in vitro into embryonic bodies that show differentiation into the three germ layers. However, both papers describing ES cells with conditional alleles of *Dicer* differ on an important point: viability of *Dicer* mutant ES cells appears to be severely compromised in one study[41] while the second paper by Kanellopoulou et al.[40] does not report this phenomenon. This striking difference remains to be explained.

The availability of conditional alleles of the *Dicer* gene in mice has allowed for analysis of Dicer and microRNA function in specific organs. *Dicer* has been targeted in T cells,[42,43] the limb mesenchyme,[44] the lung,[45] and the epidermis.[46,47] In most of these experiments loss of *Dicer* was accompanied by strong induction of apoptosis. The effects of loss of *Dicer* were relatively mild, similar to observations made in *Dicer* mutant zebrafish, while complete and timely elimination of Dicer and hence of mature microRNAs may not have been achieved in all of these experiments. Nevertheless, these experiments show that *Dicer* and consequently microRNAs are important for the proper formation and function of many mammalian tissues (see Table 1).

From the phylogenetic microRNA expression data and data of *Dicer* mutant mice and fish, a general concept of microRNA function has emerged. MicroRNAs do not seem to play a major role in early development with regard to cell fate specification or patterning of the embryo, at least in zebrafish. Rather, microRNAs are developmental perfectionists, meaning that one of their main functions seems to be to make sure that genetic programs are executed with precision, and they enforce the correct expression pattern of a broad set of genes. Therefore, one current hypothesis of a major function of microRNAs is that they make sure development proceeds smoothly resulting in a near utopian result.

microRNAs AND CANCER: ANOTHER MAGIC BULLET?

On the other hand, there is always potential trouble in paradise. microRNAs themselves can contribute to pathological disturbances, and this has already been well documented in tumorigenesis. A major theme that emerges from analysis of *Dicer* mutant mice (especially the tissue-specific ones) and flies is a role for microRNAs in apoptosis and differentiation. These are critical processes that can inhibit uncontrolled growth of cells. The process of apoptosis is highly conserved in animals and is used to eliminate unwanted cells in a highly controlled fashion. However, malfunction of apoptosis has been implicated in many human diseases including cancer, autoimmune diseases, and neurodegenerative diseases. microRNAs have been identified as regulators of apoptosis, and therefore they have become potential players in human diseases associated with defective apoptotic regulation.

Evidence for an anti-apoptotic function of microRNAs in cancer comes from analysis of B-cell lymphomas. The chromosomal region 13q31 has been implicated in tumorigenesis, including the tumorigenesis of B-cells. The 13q31 chromosomal region frequently shows amplifications in several tumor types, and one gene, *c13orf25*, was eventually identified as the potential culprit.[50] However, this gene actually contains a cluster of microRNAs, the *mir17-92* cluster (*mir-17, mir-18, mir-19a, mir-20, mir-19b* and *mir-92*). The expression of these microRNAs is indeed up-regulated in B-cell lymphomas.[51] In an animal model of B-cell lymphoma, over-expression of microRNAs of the *mir17-92* cluster dramatically enhanced c-myc induced lymphomagenesis. The onset of disease was accelerated and accompanied by a substantial suppression of apoptosis in the lymphomas.[51] In this tumor model, *c-myc* expression alone leads to the formation of tumors that are plagued by high levels of apoptosis. Expression of high levels of microRNAs of the *mir17-92* cluster suppress this apoptosis. O'Donnell et al[52] showed that c-Myc itself appears to be a key regulator of *c13orf25* and therefore of the expression of the *mir17-92* cluster. It was also shown by this group that one of the target genes of two of the microRNAs of the cluster was the cell cycle regulator E2F1. Mis-expression of E2F1 can lead to enhanced apoptosis and therefore suppressing E2F1 via microRNAs could lead to a more favorable growth versus apoptosis ratio in *c-myc* induced lymphomas. There may be additional crucial target genes of the *mir17-92* cluster since loss of *E2f1* does not enhance *c-myc* induced lymphomagenesis and does not abrogate excessive apoptosis in these tumors. Rather, loss of *E2f1* leads to a slowdown in proliferation via enhanced expression of the CDK-inhibitor p27kip1.[53] Perhaps the regulatory circuit between c-Myc, E2F1 and the *mir17-92* cluster has to be controlled very delicately in order to achieve a balanced result, and in B-cell lymphoma over-expression of *c-myc* and the *mir-17-92* cluster may tip the balance towards growth without triggering extensive apoptosis. Interestingly, in *Drosophila*, *dacapo* is a major target of microRNA regulation during proliferation of stem cells. Dacapo is the major CDK inhibitor and G_1/S regulator in Drosophila (similar to p21 and p27 in mammals). Loss of *Dicer-1* in Drosophila leads to a defect in G_1/S transition and decreased proliferation due to increased levels of Dacapo.[54] Not surprisingly, potential targets of the *mir17-92* cluster include cancer-associated genes, especially ones that are involved in the regulation of G_1/S

transition of the cell cycle, such as *cyclin D1*, *cyclin D2*, *cdk6*, *p21*, *p57*, *E2f1* and *Rb* family members.[55]

One recent paper goes even further in defining the role of microRNAs in cancer development. The *mir17-92* cluster was investigated, this time in a mouse model of colon carcinogenesis, where *c-myc* was used to trigger tumorigenic growth in *k-ras* and *p53* mutant colonocytes. Without *c-myc*, tumor growth is slow and lacks strong angiogenic potential. However, *c-myc* can overcome this lack of neo-vascularization through microRNA-induced suppression of the anti-angiogenic genes *Tsp1* and *Ctgf*.[56] Therefore, microRNAs not only can contribute to tumorigenesis via modulation of proliferation and cell death, but also by acting non-cell autonomously and enhancing angiogenesis. These papers, analyzing the relationship between c-Myc and microRNAs, have greatly extended our comprehension of how microRNAs can act as powerful oncogenes and have opened a new door for therapeutic interventions.

Other known oncogenic microRNAs are *miR-372* and *miR-373*.[57] They cooperate with oncogenic *ras* to transform primary fibroblasts. *Lats2/Kpm*—the mammalian homologue of the tumor suppressor like gene *lats/warts* (a component of the *Drosophila* Hippo tumor suppressor pathway)—is directly inhibited by *miR-372* and *miR-373*. Voorhoeve et al.[57] also presented evidence that this plays a role in human testicular germ cell tumors.

A NEW CLASS OF TUMOR SUPPRESSOR GENES FROM THE REALM OF microRNAs?

microRNAs have not only been implicated in tumorigenesis as oncogenes. The chromosomal region 13q14 is commonly deleted in many tumors including B-cell chronic lymphocytic leukemia (CLL). Absence of protein-coding genes associated with this deletion, was explained with the realization that the actual target of this chromosomal loss is a microRNA cluster. *Mir-15a* and *mir-16-1* are contained in the minimal deleted region at 13q14.[58] CLLs exhibit a reduction in *miR-15a* and *miR-16-1* gene expression and the level of these microRNAs is inversely correlated with BCL2 protein levels.[59] *bcl2* could be identified as a target gene and its modulation by *miR-15a* and *miR-16-1* was associated with sensitivity to apoptosis. Another miRNA with potential tumor suppressor activity may be *miR-17-5p*. This miRNA is able to regulate *Ncoa3* (also *Aib1* or *Src3*), an oncogene associated with the chromosome 20q12 amplicon in breast and other carcinomas.[60]

It must have come as a big surprise that Ras GTPases are also targets of microRNAs. The microRNAs involved in post-transcriptional regulation of Ras expression are *let-7* family members. Loss of *let-7* leads to failure in terminal differentiation of certain cells in *C. elegans*, although these cells continue to proliferate.[61] This is surprisingly similar to changes in tumor cells. Johnson et al. have shown the *C. elegans ras* homologue *let-60* has functional *let-7* target sites in its 3'UTR and *let-7* microRNAs can regulate Ras expression in *C. elegans* and in human cells in vitro. *Let-7* also can reduce the tumorigenic potential of lung cancer cells in vitro as measured by a colony-forming assay, and reduced *let-7* expression is associated with poor prognosis in lung cancer patients.[62] Taken together, these results give strong evidence for a tumor suppressor activity of *let-7* family members, and this is at least partially mediated by suppressing Ras activity. This also is further evidence for the notion that certain signaling pathways, in particular ras signaling, may actually be regulated and mediated not so much on the transcriptional level.[63,64] Rajasekhar et al.[64] found that Ras signaling has only a very modest immediate impact on the overall change in transcription. However, analysis of changes in the polysomal pool of mRNAs (the mRNAs that are actually translated into protein) show much more dramatic results, indicating that ras signaling mediates cellular changes via control of translation of mRNAs.

MECHANISMS OF microRNA REGULATION IN CANCER

Although there is good evidence that certain microRNAs function as oncogenes and others as tumor suppressor genes, there are other, more generalized levels of microRNA regulation in tumor cells as well. microRNA expression in tumors shows broad alterations compared to normal tissue: in general, microRNA levels are reduced in tumors.[65] One conceptual explanation for this is the idea that microRNAs are associated with a more differentiated status, predicting that each step to a less differentiated cell type should result in a loss of microRNA diversity and expression levels. microRNA expression and activity can be regulated at multiple steps. Recently, it was shown by Thomson et al.[4] that processing of microRNAs is negatively affected at the Drosha step not only during early mouse embryogenesis but also in tumors. This adds another layer of complexity to microRNA regulation in cancer in addition to chromosomal abnormalities in microRNA genes,[66,67] transcriptional activation (*mir-17-92* cluster by c-Myc) and potentially epigenetic silencing.[68]

Dicer may also contribute to the centromeric silencing via the RNAi pathway. Defective centromeric chromatin structure can result in aneuploidy. It is unclear whether loss of *Dicer* also can contribute to chromosomal instability and consequently to tumor formation. This, however, would be independent of microRNAs.

It appears that microRNAs have perplexed our somewhat overconfident assumption that we have made considerable progress in understanding cancer. Considering the number of patients dying from cancer each year in the US alone (about 500000), and the limited tools available to fight this disease intelligently, there is a lot of room for improvement. For example, microRNA expression profiles using just 217 microRNAs are superior to large mRNA expression profiles with about 16000 genes in distinguishing normal cells from tumor ones, and especially in characterization of undifferentiated tumors of unknown origin.[69]

Our rapidly expanding knowledge of miRNA function during tumorigenesis may indeed lead to novel approaches to cure cancer. It is no accident that RNAi has been suggested as a powerful therapeutic tool. However, now miRNAs themselves are implicated in the tumorigenic disease process, providing the opportunity to beat cancer with its own weapons. It remains to be seen, whether we will be able to mold the appropriate tools for this purpose and whether there will be any magic to them.

However, much can be learned in the field of cancer research from how microRNAs and transcription factors coordinate the expression of the genome during embryogenesis. Embryogenesis is like a symphony with many reoccurring themes and melodies, in which miRNAs function as metronomes to guide the orchestra of proteins to execute the score precisely according to the composer's ideas (that is evolution). In contrast, cancer represents a cacophony with misguided instruments directing and distorting the original score.

Table 2 **The MicroRNA machinery and its association with human diseases**

Disease	Gene	Mechanism	Literature
Fragile X Syndrome	*Fmr1*	recruits RISCs to specific mRNAs	Caudy et al.[84]; Jin et al.[86]
Spinal muscular atrophy	*Gemini3* and *4*	can bind to argonaute and associated with miRNAs	Dostie et al.[92]
DiGeorge syndrome	*Dgcr8*	partner of Drosha	Landthaler et al.[93]
Tourette's disease	*Slitrk1*	changes in miR–189 binding site of Slitrk1 gene	Abelson et al.[94]

SURPRISING NEW INSIGHTS INTO HOW OUR ADULT LIFE IS MANAGED BY microRNAs

microRNAs appear to be important beyond embryogenesis and tumorigenesis. For example, if we consult the expression pattern of *miR-1*, it becomes obvious that *miR-1* is much more highly expressed in the adult muscle and heart compared to embryonic and neonatal tissues.[27] Similar observations have been made in zebrafish using microarrays[21] and in mammals.[22,70] Many microRNAs show strong expression in the adult fish and their expression appears to be tissue-specific for the most part. Several reports show that animals have included microRNAs in many complex processes of the adult organism from the regulation of endocrine function to the regulation of life span. For example, the previously discussed miRNAs *miR-14* and *mir-278* have clear implications in the regulation of the fat metabolism in the adult fruit fly. In mammals, *miR-122*, a microRNA highly expressed in the liver, is involved in the regulation of plasma cholesterol levels in mice.[71] Furthermore, *mir-375* is a pancreatic islet-specific microRNA that is involved in the regulation of insulin secretion probably via inhibition of myotrophin.[72] Even the interference of microRNAs with amino acid catabolism has been demonstrated.[73] A hint for the essential function of the microRNA machinery in adulthood comes also from a study on the toxicity of high levels of short hairpin RNA in the liver. The toxicity appeared to be due to an overload of the microRNA processing system and resulted in the death of animals within two months.[74] The effects of microRNAs on metabolic regulation are more thoroughly reviewed by Krutzfeld and Stoffel.[75]

Lecellier et al. found that *miR-32* acts as an anti-viral microRNA,[76] and *Drosophila Dicer-2* has been shown to be important for innate immunity against viruses.[77,78]

Viruses themselves have developed mechanisms to suppress the mircroRNA machinery that targets them.[79] Even more intriguing, certain DNA viruses have adapted the microRNA idea and introduced novel microRNAs in their genomes[80,81] (for an overview of the interplay between viruses and miRNAs see Sarnow et al., ref. 82).

Even the response to stress on the cellular level involves changes in microRNA mediated post-transcriptional control. Bhattacharyya et al.[83] have shown that mRNAs targeted by *miR-122* can be redirected from the P-body to polysomes after stress.

Further examples of the importance of miRNA function in human disease come from studies on the fragile X syndrome gene *Fmr1* (fragile X mental retardation gene 1). The protein derived from the *Fmr1* gene is a RNA binding protein that can inhibit translation of certain mRNAs. FMR1 can recruit RISC components like argonaute proteins, Dicer and microRNAs, and facilitate contact with the target mRNAs. Through FMR1, microRNAs and/or other small RNAs may impact brain neuronal plasticity, learning, and behavior. Furthermore, FMR1 has been implicated in the control of the circadian rhythm, suggesting a possible involvement of microRNAs of the biological clock.[84-86] Table 2 gives an overview of the potential involvement of the microRNA machinery in human genetic diseases.

microRNAs IN THERAPY: NEW MIRACLES OR JUST A MIRAGE?

In the near future miRNAs will probably have been implicated in the control of almost all cellular processes. Their involvement in proliferation, apoptosis and metabolic control have already made them potential targets for the therapy of cancer and diabetes. Exciting results have been obtained in mice using "antagomirs", cholesterol-conjugated stabilized antisense microRNAs, which can be directed very specifically against a certain microRNA and inhibit its function.[71] The microRNA field can also profit from many years of experience from work with antisense technology. Several stabilized forms of oligonucleotides have been tested successfully (LNA, PNA, morpholino). However, it appears that there is still a lot of work to be done to better understand the world of small RNAs and how they can be used to cure human diseases.

GENE REGULATION: BEYOND TRANSCRIPTION AND TRANSLATION

With the realization that microRNA-mediated post-transcriptional gene expression regulation is a conserved process in almost all eumetazoa, a shift from the primary focus on transcriptional regulation to a broader more holistic view of how the expression of genes is regulated, appears possible. Additionally, our obsession with protein coding genes may eventually end since recent studies also have shown that perhaps half of the genome is actually transcribed and the vast majority of it seems not to be translated into proteins.[87,88] Importantly, it was recently shown that a noncoding RNA gene has evolved dramatically during human evolution and may have had a considerable impact on human development.[89] MicroRNAs have contributed substantially to this view of the genome and renewed interest in RNA biology. Our understanding of microRNA function has truly come a long way since their formal discovery in 1993[90,91] and undoubtedly these small molecules are likely to be at the crux of our future understanding of many human diseases and the development of novel therapeutic strategies.

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