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MicroRNA: past and present

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1. ABSTRACT

MicroRNAs (miRNAs) are ~22 nucleotide (nt) non-coding RNAs that participate in gene regulation. MiRNAs confer their regulation at a post-transcriptional level, where they either cleave or repress translation of mRNAs. Over 3000 identified mature miRNAs exist in species ranging from plants to humans, suggesting that they are ancient players in gene regulation. A relatively small number of miRNAs have been experimentally tested for their function. Of those tested, functions including cell differentiation, proliferation, apoptosis, anti-viral defense and cancer have been proposed. Improved software programs are now able to predict the targets of miRNAs in a more efficient manner, thus facilitating the elucidation of miRNA function. Furthermore, methods such as real-time PCR and microarray have been enhanced for studying miRNA expression. Using these tools, scientists are able to discover novel functions for miRNAs. It is possible that miRNAs will be revealed as having a role in virtually every aspect of gene regulation. This review guides readers through the biogenesis of miRNAs, their mechanism of action on their target mRNAs, the functional outcomes of their action on mRNAs and the current techniques to investigate these processes.

2. INTRODUCTION

From conception to death, the human body tightly regulates the activity of genetic material in order to meet demands. Gene regulation is the cellular management of the quantity and timing of the emergence of the functional product of a gene. Any step of gene expression may be modulated, from RNA transcription to protein post-translational modification. Gene regulation gives the cell control over its structure and function, and is the basis for cellular growth, differentiation and apoptosis lending to the adaptability of an organism whether it be a plant or a human.

When one thought of gene regulation in the past, he or she usually imagined such things as DNA methylation, histones or transcription factors. However, a new area of investigation is concentrating on the role of microRNAs (miRNAs) in the regulation of gene expression. MicroRNAs are small non-coding RNAs that are involved in post-transcriptional gene regulation in organisms ranging from plants to humans (1, 2). These RNAs convey their regulatory properties by either cleaving target mRNA or repressing translation (1, 3-5). Approximately one percent of the human genome is

dedicated to miRNAs and these miRNAs in turn regulate roughly one-third of all mRNAs in the human body. MiRNAs are distinguished from other forms of RNA in that miRNAs are ~22 nucleotides in length, can come from regions in the genome that are distinct from other recognized genes and have sequences that are highly conserved in related organisms (1, 6). Although miRNAs are functionally similar to short interfering RNAs (siRNAs), they are unique in their means of biogenesis. Since the discovery of the first miRNA in 1993 (7), thousands of miRNA genes have been documented in the MicroRNA Registry (8). So far, miRNAs have been experimentally identified from a wide range of eukaryotic organisms such as plants (9-12), mammals (13, 14), birds (15), fish (16) , amphibians (17), worms (18-22) and flies (23). Even more have been predicted by computational approaches by programs such as MiRscan and MiRseeker (24, 25). Although it has been difficult to assign function to miRNAs, important roles are emerging that comprise control of developmental timing, tissue growth, tumor suppression, cell differentiation and apoptosis (26-30). In this article, we will review the biogenesis, mechanisms, and known functions of miRNAs. In addition, we will discuss the methods available to investigate the expression of miRNAs and their functional targets.

3. HOW ARE microRNAS GENERATED?

MiRNAs are now considered as a general mechanism for gene regulation. The most important property of miRNA genes is that they are conserved throughout evolution, except for those found in viruses. More than half of the known mouse and human miRNAs have their homologs in pufferfish or zebrafish. The locations of miRNA genes in the genome of these organisms are variable. Some exist individually and others are in clusters that are co-expressed polycistronically. Studies of genomic positions of miRNAs in mouse and human have revealed that many miRNA genes are in the intergenetic regions. Several other miRNA genes are located in introns of coding regions, either in sense orientation or reverse orientation. This means that miRNAs can be transcribed from their own promoters or derived from the transcript product of other genes.

The biogenesis of miRNAs occurs in a stepwise fashion in the nucleus and the cytoplasm. Mature miRNAs are shortened RNAs that are the result of a series of cleavage processes that begins with a primary miRNA (pri-miRNA) (1, 2). First, pri-miRNA is processed in the nucleus into a precursor miRNA (pre-miRNA) hairpin by Drosha, a double-stranded RNA (dsRNA)-specific ribonuclease (31-33). Then, the hairpin RNAs are transported to the cytoplasm where they are digested into lengths of ~22 nt by another dsRNA-specific ribonuclease called Dicer (34). This mature form of miRNA is now available to bind to a RNA-Induced Silencing Complex (RISC) in order to repress translation or direct cleavage of target RNAs (35). The following breaks down this process from a primary to an active miRNA.

3.1. Transcription of primary microRNAs

The pri-miRNAs are transcribed as long, double-stranded unstructured precursors with a 5' cap and a 3' playa tail and are dependent on regulatory machinery presumably as encoded RNA destined for translation into protein (36, 37). Many genes for miRNAs are separated from other genes, but some (~ one-third) are found within introns of protein-coding genes where they are likely controlled by a shared promoter (38). As for transcription of miRNAs genes, it remains unclear as to which RNA polymerase is responsible for the transcription: there are two polymerases. Pol II is responsible for the transcription of protein-coding genes, small nucleolar RNA genes and some small nuclear RNA genes (39-41). On the other hand, pol III transcribes small non-coding RNAs including tRNAs, 5S rRNA, some snRNAs and other small RNAs (42). Several observations have suggested that pol II may be the most important RNA polymerase engaged in miRNA gene transcription. First, the pri-miRNAs are longer than the ~70 nt pre-miRNAs and sometimes can be several thousand bases long. For example, pri-miR-21 is ~3433 nt in length, therefore, it is too long for pol III- mediated transcription (37, 43). Second, it is believed that the pri-miRNA gene sequences are flanked by promoters in the 5' region that are able to transcribe heterologous mRNAs (43). Third, stretches with more than four U's, which terminate the transcription of pol III, widely exist in pri-miRNA sequences (44). Forth, human pri-miRNAs are polyadenylated and capped, which are the unique properties of pol II gene transcripts (45). However, some new evidence indicates that, in some cases, miRNAs can also be transcribed by pol III. In the genome of mouse gamaherpesvirus 68 (MHV68), miRNAs are embedded in the pol III tRNA primary transcripts and are ended with a stretch of oligo(T) (46). The transcription of these miRNAs is largely unregulated as they are under the control of an unregulated tRNA-specific pol III promoter. From these observations we can speculate that both pol II and pol III may transcribe miRNA genes depending on the species and the evolutionary origins of these miRNAs.

3.2. Processing of primary microRNAs by Drosha

Ribonucleases serve as the post-transcriptional regulators that participate in the processing and decay of RNA. Thus, the rates of maturation and decay, along with transcription frequency, establish the steady-state level of an RNA. In the cell's nucleus, a specific ribonuclease of the RNase III endonuclease family called Drosha enzymatically cuts the pri-miRNA into a smaller (~70 nucleotides) product (31). Drosha is able to do this with the help of a cofactor called DGCR8 or Pasha, which is a dsRNA binding protein that dimerizes with Drosha (47). Drosha is composed of two tandem RNase III domains, a dsRNA binding domain and an N-terminal segment (48). It is a dsRNA specific endonuclease that produces the 2-nucleotide 3' overhang at the cleavage site (49). Drosha cleaves both strands of pri-miRNA into an imperfect stem-loop structure, which predetermines one end of the mature miRNA.

Mutagenesis studies reveal that the enzymatic activity of Drosha is dependent on the cis-acting elements flanking the cleavage site, the length of the terminal loop and the stem structure (31, 50). However, the internal loop, or bulge, has little effect on the efficiency of Drosha.

Recent knock-down studies show that Drosha cannot work without its partner, DGCR8 (51). Human Drosha is in two multi-protein complexes with one being larger and containing several classes of RNA-associated proteins. The other smaller multi-protein complex is called a microprocessor, containing both Drosha and DGCR8. The deletion of DGCR8 in humans has been shown to cause DiGeorge syndrome, a developmental disease characterized by specific cardiac malformations, facial abnormalities, and certain endocrine and immune anomalies (47). Further *in vivo* and *in vitro* experiments show that this Drosha-DGCR8 microprocessor is necessary and sufficient for the processing of pri-miRNA to pre-miRNA (52). The relative role for the larger Drosha multi-protein complex remains to be unknown.

3.3. Export of precursor microRNAs to the cytoplasm

After being cleaved by Drosha, pre-miRNA is transported to the cytoplasm by Exportin-5, a nuclear export factor, in a Ran-GTP dependent manner (53, 54). This type of nuclear export factor is known to shuttle transfer RNAs, short hairpin RNAs and ribosomal RNAs to the cytoplasm (55). Exportin-5 was first examined for its involvement with the nuclear transport of adenovirus VA1, a viral non-coding RNA with an 18 base pair stem (56). The binding of Exportin-5 to pre-miRNA is specific because a stem must be larger than 14 base pairs with a base-paired 5' end and a short 3' overhang in order for Exportin-5 to bind efficiently (57). In addition, the binding of Exportin-5 to the pre-miRNAs *in vitro* prevents its degradation by a bacterial exonuclease, suggesting that the export factor provides protection for the pre-miRNA from nucleases as it is being transported out of the nucleus (57).

3.4. Maturation of microRNA in the cytoplasm

Once the pre-miRNA is transported into the cytoplasm, it is cleaved into an imperfect dsRNA duplex designated miRNA: miRNA*. Essentially, an extension of the stem-loop is cut off to yield the active ~22 nt miRNA. This process is carried out by Dicer, an RNase III endonuclease, which defines the other end of the mature miRNA (37, 58). Dicer is an evolutionarily conserved enzyme in mammals, worms, fungi, flies and plants. It is composed of a helicase domain, a Piwi-Argonaute-Zwille (PAZ) domain, two RNase III motifs, a Domain of Unknown Function (DUF283) and a dsRNA binding domain (dsRBD) (58, 59). Dicer forms an intramolecular dimer with its two RNase III domains, which generates an active catalytic site at the interface of the RNase III dimer for single dsRNA cleavage. This process is unlike Drosha because Dicer's second RNase III domain contains substitutions at a critical active site, which makes it partially inactive (60). The crystal and NMR structures of the PAZ domain have been solved (61, 62). It contains a five-stranded central β-barrel, which is a topological variation of the oligonucleotide/oligosacarride-binding (OB) fold. The OB structure predominantly functions in single stranded nucleic acid binding. The PAZ domain has a weak but consistent affinity for nucleic acids. Moreover, it has specificity for the 3' two-nucleotide overhang resulting from RNase III cleavage (62). This suggests that the PAZ domain may have a major contribution to the substrate specificity of Dicer.

3.5. Species variation in microRNA maturation

There are two Dicer homologues in *Drosophila melanogaster*, Dicer1 and Dicer 2 (63). Dicer1 plays an important role in pre-miRNA processing as Dicer1 deficiency blocks this process. A dsRNA binding protein, R3D1, forms a stable complex with Dicer1 and is necessary for pre-miRNA processing (64). In contrast, Dicer2 does not have an effect on miRNA maturation but rather affects short interfering RNA (siRNA) production. It forms a complex with the dsRNA binding protein, R2D2, that initiates RISC assembly and enhances mRNA degradation mediated by RISC (63, 65, 66). The orientation of this complex determines which siRNA strand is assembled into RISC (67).

The maturation of miRNA in plants is much different from that in animals as no Drosha homologue has been identified in plants thus far (12). However, four Dicer homologues exist in the flowering plant, *Arabidosis Thaliana.* Two of these Dicer-like proteins are likely localized in the nucleus (68, 69). Dicer-like protein-1 (DCL1) possibly performs both Drosha and Dicer-like activities for miRNA maturation inside the nucleus (70). Interestingly, a miR162 target sequence is predicted near the middle of the DCL1 mRNA, suggesting that DCL1 is dependent on negative feedback regulation by a miRNA (71). Therefore, *A. Thaliana* as well as other plants that have existed over 100 million years utilize redundant regulatory mechanisms for processing miRNA (72). The complexity of miRNA maturation in animals is likely an evolutionary byproduct.

Because cellular miRNAs have become increasingly complex in mediating post-transcriptional regulation, viruses have also evolved in order to successfully infect cells. Few studies have reported on the maturation of viral miRNA; however, it is generally thought to be similar to that of the host cells. Because miRNAs act at the mRNA level, it is not likely that viruses that possess RNA as their genetic material would encode miRNAs. The more probable choice are DNA viruses, such as herpesviruses, that establish long term latent infections in cells. Other candidates include lytic DNA viruses like the human adenovirus. Indeed, miRNAs have been found in herpesviruses as well as human adenovirus (46, 73-75). The pol II-transcribed product of most viral encoded miRNA genes is processed in the nucleus by Drosha and transported by Exportin-5 to the cytoplasm where it is processed by Dicer (46, 76, 77). One of the arms of the processed miRNA is incorporated into RISC (78). This maturation process may be not true in the case of MHV68, since MHV68 pre-miRNAs have an unusual short hairpin structure, which means the maturation and export mechanism may be different from those of the host cells (46). Furthermore, studies of the human adenovirus show that the viral miRNA has the ability to mimic the stem loop structure of the host miRNAs in order to bind and sequester Dicer (75). The same study demonstrates that Dicer not only binds to the viral pre-miRNA, but also

cleaves it to yield a ~22 nt mature miRNA. Although these reports provide evidence that viral miRNAs exist largely at the expense of the host cell, no host cell mRNA targets for the viral miRNAs have been identified. Instead, identified targets are viral mRNAs that either allow the virus to remain in the latent stage or allow the virus to become lytic without being recognized by the host's immune system (76, 79). If host mRNAs are found to be targets, they will most likely be involved with the innate or adaptive immune systems of the host (80).

4. HOW DO microRNAS WORK?

4.1. Formation of RISC

After pre-miRNA is processed into a miRNA-miRNA* duplex by Dicer, one of the RNA strands is incorporated into RISC for target recognition (81). RISC has strand bias and always incorporates the miRNA strand with the less stable 5' end in comparison with the miRNA* strand (82). The miRNA* strand is degraded in most cases . This strand bias may be the result of the relative ease of unwinding from one end of the duplex. A helicase likely carries out multiple trials from both ends in order to unwind the duplex. Occasionally, these trials lead to unwinding at the easier end of the complex, rendering a strong bias for the easier end. This is supported by the finding that in rare cases where miRNAs have 5' ends with similar stability, both strands are incorporated into RISC (81).

RISC is composed of Dicer, Argonaute (AGO) and other non-specified proteins (83). AGO proteins bind to either miRNAs or siRNAs to create the core of the complex. They have both Ago and Piwi domains. The Ago domain, which is a PAZ domain, binds to the 2-nt overhang at the 3' end of RNA duplexes (62). The Piwi domain mediates interactions between Argonate proteins and Dicer (67). Different Ago paralogs exist across species, and the Ago-associating proteins are also variable among species. Furthermore, variants of the AGO protein within the same animal can have different functions. For example, among RISC with any of the 4 human AGO proteins (AGO1-AGO4), only AGO2-associated RISC can direct the cleavage of target mRNA (84). Drosophila AGO2 acts to cleave target mRNA, while AGO1 functions in translational repression (85). Some of the other proteins that associate with AGO proteins are the Vasa intronic gene *(VIG*), an endonuclease like protein (TSN-1) and a homolog of fragile X mental retardation protein (dRXR) (86, 87). Most AGO-associated proteins have unknown functions, but some are required to mediate mRNA cleavage (88). It is likely that the different AGO homologues along with the variable associating factors allow for different subtypes of RISC in order to provide a specific response to a particular siRNA or miRNA. RISC has many diverse functions in both siRNA and miRNA mechanisms. It acts as an effector complex in mRNA cleavage, translation suppression and heterochromatin formation (89, 90). The details for the molecular mechanisms that make RISC work are still not clear, however, some observations have been made that will be discussed below.

4.2. Translational repression

MiRNA-associated RISC can repress mRNA translation in a couple of ways. MiRNAs in animals mostly suppress translation of their target mRNAs due to an imperfect base-pairing within 3' untranslated regions (UTRs) (91, 92). By binding to the 3' UTR of the mRNA, the miRNA has the ability to inhibit translation by directly interfering with translation initiation factors or by disrupting polyA tail function (3, 93, 94). However, polyribosomes co-purify with RISC, indicating that RISC may also interrupt the continuation of translation by forming a stable complex with polyribosomes (95, 96).

The distinction between translational repression and mRNA cleavage mediated by miRNA relies on the degree of complementarity between the miRNA/siRNA and its target. Plant miRNAs function in mRNA cleavage due to their near complete complementarity in base-pairing to their target mRNA (97-100). When mammalian miRNAs have high enough complementarity with their target mRNA, they perform mRNA cleavage instead of translational repression as in the case of *miR-196* and its target Hoxb8 (100, 101). Although most plant miRNAs cleave and destroy their target mRNAs with nearly perfect complementarity between them, one plant miRNA, *miR-172,* acts as a translational suppressor (99, 102). Furthermore, when the base-pairing between siRNA and its mRNA target does not have strong complementarity, siRNA can perform translation repression just as miRNA, suggesting that miRNAs and siRNAs are functionally interchangeable (103).

4.3. mRNA cleavage

The perfect base-pairing is thought to be the critical feature of miRNA-mediated mRNA cleavage, but it is not always sufficient in plants, suggesting the need for supplementary catalytic activity by RISC (102). Regardless of the species, the perfect match between the miRNA and target mRNA is required for efficient cleavage, especially considering the precise location of the cut between residues 10 and 11 of the miRNA (99, 104, 105). Once the mRNA is efficiently cut, the miRNA has the ability to cut other target mRNAs or guide mRNAs to mRNA processing bodies (P-bodies) for subsequent degradation (97, 105).

4.4. mRNA degradation

MiRNAs can also act upon their target mRNAs by increasing the rates of mRNA degradation in a RISC-independent manner. By transfecting *miR-1* and *miR-124* into human cells and using microarray, Lim *et al.* show that a significant number of mRNAs are downregulated in accordance with the miRNA expression (106). Another study reports that *miR-16* contains an AU-rich sequence complementary to the AU-rich element (ARE) in the 3' untranslated region of unstable mRNAs, a sequence that is known to increase mRNA decay rates (4). The finding suggests that miRNAs are required for ARE-RNA turnover. Furthermore, Bagga *et al.* demonstrates that the expression of two of the earliest identified miRNAs, *let-7* and *lin-4*, significantly decrease the levels of their target mRNAs

(107). All three studies provide evidence that miRNA can directly affect the turnover of their target mRNAs.

MiRNAs are also associated with P-bodies. Cytoplasmic P-bodies are compartments in the cell that contain decapping enzymes and exonucleases for the degradation of mRNA. RISC, particularly AGO proteins, co-localize with P-bodies. Recent studies show that the localization of targeted mRNAs to the P-bodies are miRNA-dependent (108-110). It is still not clear what functional capacity RISC possesses in P-bodies. Although P-bodies are primarily known for retaining targeted mRNAs for degradation, they contain several factors that are associated with translational repression (111). Once the targeted mRNAs are sequestered in the P-bodies, they generally undergo deadenylation by removal of the 3' polyA tail, decapping by Dcp1/Dcp2 and degradation by an Xrn exonuclease (111, 112). Deficits in the expression of the exonuclease leads to an inefficient RNAi response (113, 114). The miRNA-mediated degradation pathway is now known to be dependent upon the GW182 protein that interacts with the Piwi domain of AGO (115). Taken together, miRNAs can contribute to mRNA turnover by transporting mRNAs to P-bodies.

5. WHAT DO microRNAS DO?

The previous section described the mechanisms of miRNA-mediated gene regulation. In this section, we will briefly discuss some of their physiological functions. MiRNAs have been implicated in biological processes ranging from developmental timing to apoptosis. As scientists discover the role of miRNAs in even more processes, it will become clear as to just how important these tiny RNAs really are.

5.1. Developmental timing

Most of the knowledge of the biogenesis and function of miRNAs stems from the paradigm provided by the discovery of *lin-4 and let-7*, which were identified as miRNAs implicated in the function of *C. elegans* developmental timing (7, 116). *Lin-4* and *let-7* bind to multiple conserved sites in the 3' UTR of the *lin-14* and *lin-41* transcripts, respectively, through direct but imprecise base-pairing, thus inhibiting translation into protein (7, 91, 117). In *C. elegans*, the down-regulation of the protein LIN-14 at the end of the first larval stage initiates the second larval stage (7). Lin-41, on the other hand, regulates the developmental transition from the last larval stage to the adult stage (117). Computational analysis reveals that *let-7* is evolutionary conserved throughout metazoans (118). *Lin-4* and *let-7* also regulate two other genes, *lin-28* and *lin-57,* respectively (119, 120). *Lin-28* encodes a RNA-binding protein that is important for neuronal differentiation of embryonal carcinoma cells (121) while *lin-57* encodes a protein responsible for the terminal differentiation of the hypodermis in *C. elegans* (120). Since the original C. elegans experiments, the regulatory abilities of *lin-4* and *let-7* have been extended to flies and mammals (122). In mice, these miRNAs inhibit expression of *lin-41*, which is involved in key developmental events such as limb formation (122). Studies of three miRNAs in *Drosophila*, *let-7*, *miR-125* (the *lin-4* homolog) and *miR-100*, not only show their upregulation during major points of development but also demonstrate their requirement of a concurrent expression of a hormone in order to be functionally expressed (123). Important mRNA targets responsible for developmental timing have also been found in *Arabidopsis thaliana,* suggesting that miRNA regulation in morphogenesis is a primitive mechanism (124, 125).

5.2. Cell proliferation and differentiation

In addition to guiding timing events, miRNAs have also been established as potent controllers of cell proliferation and differentiation. Although the division of cells is imperative for the growth of an organism, it can also be detrimental when occurring at inappropriate times. The latter is the hallmark of cancer, and several miRNAs have been shown to be upregulated in tumors, a concept that will be discussed later along with miRNA-associated diseases. It is important to understand the significance of cell division during organismal growth beginning with stem cells. Mutation studies of *Drosophila* show that disruption of miRNA processing causes stem cells to be locked between the G1 and S phases, thus halting division (126). A neuron-specific miRNA, *miR-132*, is a target of the transcription factor, cAMP-response element binding protein. It regulates neuronal growth by decreasing the levels of a GTPase-activating protein (127). Another brain-specific miRNA, *miR-134*, is expressed in the synapto-dendritic compartment of rat hippocampal neurons, where it is capable of down-regulating Limk-1, a protein responsible for spine development (128). Regulatory roles of miRNAs are not limited to the brain. Adipose cell differentiation has been shown to be partially controlled by the expression of *miR-143* (30). Also, *miR-1* and *miR-133* are important regulators of skeletal muscle proliferation and differentiation (129). Because cell growth and differentiation are such dynamic biological processes, it is no wonder that miRNA with its specific and fast-acting regulatory ability is vital for shaping these processes.

5.3. Apoptosis

Apoptosis, programmed cell death, is an integral part of animal tissue development. Apoptosis is an evolutionarily conserved process that allows animals to remove cells that are useless or that are detrimental to survival. Once apoptosis is activated, caspase proteins cleave both the structural and functional elements of the cell. Therefore, cell death and survival depend largely on the control of active caspases in the cell. Because caspases are ubiquitous, it makes sense that miRNAs would play a role in their regulation. Indeed, in the *Drosophila* eye, the absence of *miR-14* leads to an increase in the cell death effector, Drice, suggesting that *miR14* is an inhibitor of apoptosis (130). Likewise, the *bantam* gene encodes an miRNA that when over-expressed, suppressed apoptosis in the *Drosophila* retina. One of the targets for *bantam* was identified as the pro-apoptotic gene, *hid*, which possesses sequences of complementarity to *bantam (131).* It has been known for quite some time that viruses must prevent apoptosis in order to survive in the host cell. Recently, it has been discovered that the herpes simplex virus-1 inhibits apoptosis through a latency-associated miRNA (miR-*LAT*)

that modulates TGF-β signaling (132). By using miRNAs instead of proteins in the inhibition of apoptosis, viruses are able to survive as well as evade immune detection. As functional studies of miRNA continues, the list of targets involved in apoptosis will likely grow radically.

5.4. Disease

Although miRNAs have been established as being vital for animal development, they are also associated with diseases when their repressing activities are compromised. Some of miRNAs, including *miR-143* and *miR-145,* have been identified as possessing tumor suppressor activity. Thus, their down-regulation leads to tumorogenesis (133, 134). The exact targets for these miRNAs have not been elucidated, but they are likely genes that regulate the cell cycle. Another study found that *miR-15* and *miR-16* directly suppresses the *BCL2* oncogene (135). Furthermore, the previously discussed miRNA, *let-7,* has been linked to RAS, a potent activator of cell transformation (136). Several different miRNA clusters have also been associated with the *MYC* oncogene (137-139). Although the majority of miRNAs are down-regulated in cancers, some including *miR-21* are up-regulated due to their anti-apoptotic effects (140). Whether *miR-21* has a direct role in cancer progression or is simply differentially modulated in tumors still needs to be experimentally clarified.

Because miRNAs are involved in cancer, they may serve as important cancer prevention genes. Indeed, experiments are already underway in model systems to inactivate miRNAs that may serve as oncogenes (141-143). However, the new era of therapeutic targeting of miRNAs is not limited to cancer. A recent study of *miR-375*, a pancreatic-specific miRNA that regulates insulin secretion, suggests that miRNA therapies may also be applicable to diabetes (144). As more miRNAs are linked to diseases, it is possible that this approach can be applied to virtually any organ system in the body.

5.5. Anti-viral defense

Albeit miRNAs are implicated in diseases caused by malfunctions in the cellular machinery, they also serve an important role in preventing diseases caused by viruses. Scientists studying plants first proposed miRNAs as being able to induce post transcriptional gene silencing of viral mRNAs (145, 146). In plants, miRNAs have anti-viral capability with short-lived effects because evolved viral factors eventually inactivate the miRNAs (147). In fact, many viruses have the capability to evade silencing by the host, but some viruses are better adapted for evading cellular machinery than others. In humans, for example, adenovirus can block host miRNA biogenesis, thus squelching the very anti-viral miRNAs that are meant to stop adenovirus replication (148). Also, tissue culture experiments show that the primate foamy virus type I (PFV-1) can escape silencing by *miR-32* with a silencing suppressor protein called Tas (149). These observations suggest that host miRNA-mediated defense cannot always overcome viral attacks. However, these experiments do not account for the possibility of defense responses mounted by multiple miRNAs working together. A study of the hepatitis C virus demonstrates that the introduction of multiple siRNAs targeted to different areas of the viral genome prevents the virus from escaping siRNA-silencing (150). Future experiment with miRNAs will likely produce similar results, considering that miRNAs are functionally similar to siRNAs. Due to the accelerated mutational activity of viruses, miRNA-mediated anti-viral therapies are ideal. After all, the effectiveness of small RNAs are based on sequence, making the development of new therapeutic small RNAs expeditious, which is a far cry from conventional drug-based therapies.

6. HOW DO SCIENTISTS STUDY microRNAS?

6.1. Expression studies of microRNAs

One of the prominent characters of miRNAs is that their expression is spatially and temporally regulated. Many miRNAs are highly expressed in certain organs or cell types and some are only expressed in certain stages during development. These regulations in expression patterns obviously coordinate with their functions; therefore, much research has concentrated on expression studies of miRNAs.

The technology of miRNA expression detection ranges from simple to complex. One popular method use small RNA cloning and sequencing to identify miRNA in tissues and cells (2, 6, 16, 19, 20, 23). Although this method has potential to find novel miRNAs, the rates of discovery depend on the expression levels of the miRNAs. In general, cloning methods are not sensitive enough and not suitable for high throughput miRNA expression profiling. Furthermore, it is possible that miRNAs have bias at various steps including ligation, reverse transcription, amplification and cloning. By far the most reliable technique to detect the expression of miRNA remains to be Northern blot. After all, results from all other methods are always validated using Northern blots. For example, Sempere and colleagues made an extensive effort to uncover the expression profiling of mammalian miRNAs using Northern blots and showed that some miRNAs are specifically expressed or highly enriched in certain organs (151). In situ hybridization has also been a reliable technique for evaluating the relative expression level as well as temporal and spatial patterns of miRNAs (152).

MicroRNA microarrays have been widely used to analyze miRNA expression. Krichevsky and colleagues used a membrane spotted with antisense sequences to mature miRNAs (153). The RNA samples were end-labeled with gamma-^{32}P and hybridized to an oligonucleotide array. Of the probed miRNAs, 20 percent were significantly changed during mammalian brain development. In most of the miRNA microarray platforms, glass slides were utilized for spotting antisense or sense probes. There are many subtypes of this technique. They are different in several respects such as probe choice, RNA sample processing and detection method. Some researchers use Cy3 or Cy5 labeled cRNA transcribed from cDNA of miRNA to hybridize with chips (154). Others have developed their own microarray systems for miRNA

detection using cDNAs from adapter ligation and reverse transcription of miRNAs (38, 155-158).

There are compelling advantages for using miRNA microarrays. They are very sensitive and, more importantly, high throughput. In some microarray designs, miRNAs with concentrations as low as the fempto-mole range can be detected (158). Also, microarrays are less time-consuming compared to Northern blots. However, the challenge in using microarrays is data analysis. One potential problem is data normalization, where housekeeping genes are not suitable for data normalization. Unlike DNA microarrays, in which there are between 10 to 30 thousand probes on the slide, miRNA microarrays only contain 200 to 300 probes. Furthermore, the overall miRNA expression levels may be variable in different tissues. Therefore, global normalization used for DNA microarays may not be appropriate in some cases.

Real-time PCR has been adapted to monitor miRNA expression. One such adaptation uses stem-loop RT primers that are more efficient and specific than conventional primers (159). It is now possible to analyze over 300 different gene profiles, which is comparable to the high throughput of microarrays (160). Jiang and others successfully profiled the expression of 222 miRNA precursors in 32 cancer cell lines using real-time PCR (161). Their results show that some miRNA precursors have significantly higher or lower expression in certain cell lines. They also report that the expression of pre-miRNA parallel that of mature miRNA in most of the cells that they tested. Another study used real-time PCR to evaluate the expression profiling of several mature Arabidopsis miRNAs, where as little as 100 picograms of total RNA was used (162). The increased sensitivity of real-time PCR surpasses that of both Northern blots and microarrays, making this technique desirable.

6.2. Function studies of microRNAs

Functional studies of miRNAs can be carried out by several approaches. The first approach is forward genetics where the study starts with a phenotype and moves toward the identification of a miRNA (7, 131, 163). The second approach is reverse genetics where the study starts with a miRNA and assays the effect of the gene's disruption (138, 164). Since most miRNA research begins with the identification of the miRNA, another important approach to study miRNA function is to predict the miRNA's target gene(s) by computational algorithms and experimental validation.

6.2.1. Target prediction

It is crucial to identify miRNA targets in order to uncover the functions of miRNAs during in normal physiological and pathogenic conditions. As mentioned before, most plant miRNAs have nearly perfect complementarity with their target mRNA sequences leading to target mRNA cleavage, which makes the target identification much easier. However, identification of target sequences in animal genomes is impossible by standard sequence comparison because miRNAs are very short in length and their complementarity to target sequences is imperfect (165, 166). For this reason, computational approaches for finding animal miRNA targets is very useful. Currently there are several web servers that contain computationally predicted targets for miRNAs across many species. Among them is miRanda software (www.microrna.org) (167). The program uses the miRanda algorithm to identify potential binding sites and then checks the potential target site in the 3' UTR to see whether the sites are conserved in orthologous transcripts. Another algorithm, PicTar, predicts miRNA targets in vertebrates and *Drosophila* species and has the ability to predict targets for single miRNAs and for combinations of miRNAs (http://pictar.bio.nyu.edu/) (168). The TargetScan alogarithm identifies mRNAs with conserved pairing to the 5' region of the miRNA and assesses the quantity and quality of these complementary sites (http://genes.mit.edu/targetscan) (165). These programs have allowed for the creation of target databases that may potentially facilitate functional studies for miRNAs. One such database for predicted targets is TarBase (http://www.diana.pcbi.upenn.edu/tarbase), which contains target mRNAs tested in human, mouse, fruit fly, worm, and zebrafish (169). Another database is Argonaute (http://www.ma.uni-heidelberg.de/apps/zmf/argonaute/interface/).

6.2.2. Target validation

After finding potential targets for miRNAs, the targets need to be validated by experimental methods. To test whether the target mRNA is cleaved by the miRNA, the target mRNA is reverse-transcribed, cloned and sequenced. The cleavage site should be at the site predicted according to the miRNA: mRNA duplex (105, 170). To test whether the target is inhibited by translational repression, several methods have been used. The 3' UTR segment with predicted complementary sites or those with point substitutions that disrupt the base-pairing are fused with a reporter gene. The disruption of the base-pairing abolishes the translation inhibition of the target mRNA expression. The correct 3' UTR segment with the complementary sites is used concurrently as a control (102, 171). Another method uses RNA inhibition to repress the activity of the miRNA to see if the effect on the target mRNA is real (129).

6.3.3. Over-expression and down-regulation of microRNAs

Studies also over-express miRNAs to elucidate their effect on their target mRNA and their physiological consequence on the cell (144, 172). Over-expression of the miRNAs should significantly reduce the expression of the target mRNA. To observe the reverse effect, miRNAs are blocked by a 2'-O-methyl oligoribonucleotide complementary to the miRNA (173, 174). A screen of miRNAs involved in cell growth and death has been reported using a library of these modified oligonucleotides (175). Krutzfeldt et al. developed a new method to silence miRNAs *in vivo* with "antagomirs" (142). Instead of using antisense 2'-O-methyl oligoribonucleotides, they designed chemically modified, cholesterol-conjugated antisense RNA. Whether the miRNA is over-expressed or silenced, the target protein should have coincident expression with

the miRNA. If the miRNA is over-expressed, the expression of target mRNA should be depressed. Conversely, if the miRNA's function is blocked, the translation inhibition should be released.

7. CONCLUSIONS AND PERSPECTIVES

Since the discovery of the first miRNA in 1993, remarkable progress has been made in the area of identification, high throughput miRNA expression profiling, computational target prediction and experimental confirmation. With the increasing knowledge through observation and confirmation of more miRNA-target relationships, the empirical parameters for target recognition will be modified and made increasingly powerful and unambiguous. However, questions still linger in the miRNA world. What are the exact mechanisms for miRNA-mediated cleavage of target mRNAs? What other proteins are involved in miRNA-mediated gene regulation? Most importantly, can synthetic miRNAs be used as a therapeutic intervention to diseases? These questions and many more will be the focus of future research for many laboratories.

In the past few years, the understanding of miRNA function has increased dramatically. How widespread is the function of miRNAs? Perhaps clues will come from experiments that disrupt the function of Dicer homologues, AGO family proteins or other important factors such as GW182 that have principal functions in miRNA function (176-178). Although thousands of miRNAs have been identified in 39 species, only a small percentage of miRNAs have been tested for their function. Finding new functions for miRNAs is the new frontier of molecular science. Many suspect that miRNAs will be revealed as the most important players in gene regulation: a tall order for a tiny string of nucleotides.

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Abbreviations: miRNA: microRNA, nt: nucleotide, siRNA: short interfering RNA, pri-miRNAs: primary miRNAs, MHV68: gamaherpesvirus 68, pre-miRNA: precursor miRNA, dsRNA: double-stranded RNA, PAZ: Piwi-Argonaute-Zwille, DUF283: domain of unknown function 283, OB: oligonucleotide/oligosaccharide-binding, RISC: RNA-induced silencing complex, AGO: Argonaute, VIG: Vasa intronic gene, P-bodies: mRNA processing bodies, UTR: untranslated region, PFV-1: primate foamy virus-1.

Key Words: MicroRNA, Gene Regulation, Gene Silencing, RNA interference, 3' untranslated regions, translation repression, mRNA Turnover, Cell Division, Development, Differentiation, Apoptosis, Review

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