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Recent developments in understanding the mechanism and functions of microRNAs

Suhail Muzaffar[1*], Karishma Bisht[2]

[1] Centre for Genome Research, Department of Microbiology and Biotechnology Centre, The M. S. University of Baroda, Vadodara-390002, India.
[2]Division of Genetics, ICAR- Indian Agricultural Research Institute, New Delhi-110012, India.

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ABSTRACT

Based on their mechanism of action and biological function, several classes of small RNAs have come into the limelight in the last two decades. These small RNA molecules generally belong to three main categories: short interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs). miRNAs and siRNAs are distinguished primarily because miRNAs are endogenous in nature and are expressed by an organism's own genome, whereas siRNAs are exogenous in origin and derived mainly from the viruses and transposons. The first miRNA, lin-4, was discovered in 1993 as an endogenous regulator of genes that control developmental timing in *Caenorhabditis elegans*. miRNAs are coded by both plant and animal genomes and their transcription is typically performed by RNA polymerase II. MicroRNAs repress the expression of many genes by accelerating messenger RNA degradation as well as translational inhibition, thereby reducing the level of protein. Due to their involvement in various diseases like cancer, miRNAs have been a focus of scientific research for their potential as a new generation of drugs. The recent findings in miRNA research have been summarized in this review to add new dimensions to miRNA mechanism and functions.

1. INTRODUCTION

MicroRNAs (miRNAs) are small, evolutionary conserved, non-coding RNA molecules which mediate the post-transcriptional gene silencing in plants, animals, and some viruses. These ~22 nucleotide molecules repress the expression of various genes by messenger RNA degradation or by inhibiting the mRNA translation [1]. miRNAs look similar to small interfering RNAs (siRNAs), except that siRNA are exogenously introduced into the cell while as miRNA are endogenously produced from the regions of RNA transcripts that fold back on themselves and generate short hairpin structures [2]. miRNAs are well conserved throughout the plant and animal kingdom and are of high evolutionary significance. The human genome has been estimated to encode more than a thousand miRNAs that can target the expression of nearly 60% of the genes [1].

Dysregulation of miRNAs has been linked to different human diseases including cancer, cardiovascular diseases, metabolic disorders and viral infections [3].

In this review, we have summarized the existing information related to the discovery, mechanism, functions, and dysregulation of miRNAs.

2. DISCOVERY OF miRNA

The process of RNA interference was largely unknown until Andrew Fire and Craig C. Mello discovered that the double-stranded RNA (dsRNA) is a potent trigger for gene silencing [4]. They injected a dsRNA 742-nucleotide segment of *UNC22* gene into the body of an adult *C. elegans*. *Unc22* codes for a myofilament protein and the decrease in *Unc22* expression causes a characteristic twitching phenotype. The dsRNA injected nematodes showed the normal twitching, but the progeny showed a strong twitching phenotype suggesting the silencing in *Unc22* (Fig 1). They also demonstrated the sense strand silencing observed by Guo and Kemphues was actually due to the double-stranded RNA contamination in the samples used in the experiments. Fire and Mello were awarded the 2006 Nobel Prize in Physiology or Medicine for the discovery of RNA interference.

** Corresponding Author*
Suhail Muzaffar, National Centre for Biological Sciences
GKVK Campus, Bellary Road Bangalore 560065 INDIA.
Email: suhail.bt @ gmail.com; Phone: +91 9898726492

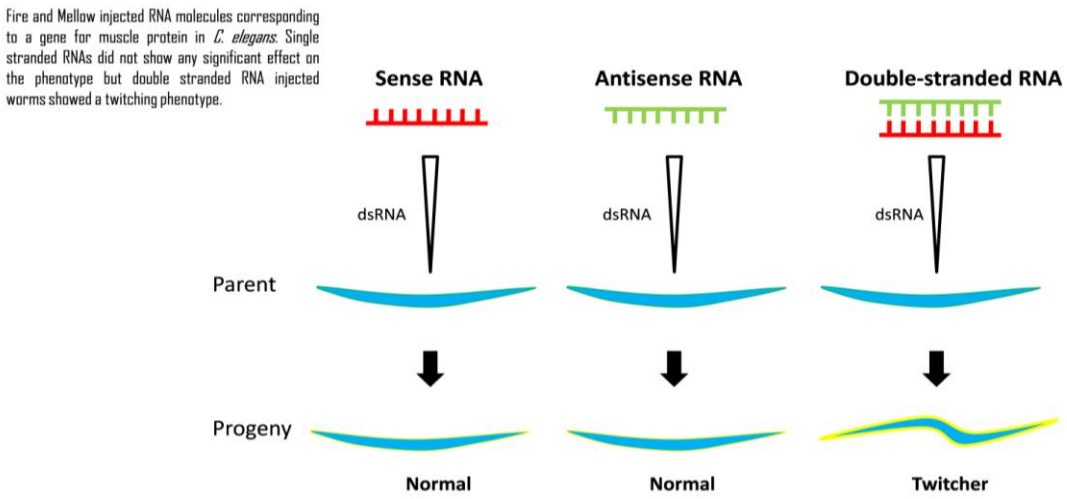

Fig. 1: Discovery of RNA interference**.** Andrew Fire and Craig Mello injected double stranded RNA molecules encoding a muscle protein *Unc22* in *C. elegans* and observed that the worms developed a peculiar twitching phenotype. This behavior was caused by the silencing of the native Unc22 gene.

Several proteins involved in the process of RNAi were discovered from the genetic screens in *C. elegans* [5]. Most of the proteins involved in the dsRNA-mediated gene silencing in *C. elegans* were found to have homologs in *Drosophila*, Fungi, and Plants, which established a common basic mechanism of RNAi. In 2001, Zamore and his colleagues observed that 21-23 nt RNAs produced from dsRNA in cell extracts of *Drosophila* could serve as the silencing trigger for RNAi in cell extracts without dsRNA treatments [6]. Later it was found that these 21-23 nt small RNAs are generated by a ribonuclease present in the RNA-induced silencing complex (RISC) [7]. Dicer, a highly conserved RNase III enzyme involved in the cleavage of small RNAs was first cloned in 2001[8]. One of the exciting observations in gene silencing was that naturally occurring micro RNA (miRNA) are also processed by Dicer and function through the common RNAi machinery [9, 10]. In 1993, the first miRNA Lin-4 was discovered in *C. elegans* in the laboratories of Victor Ambros and Gary Ruvkun [11]. lin-4 miRNA specifically binds to multiple target sites in the 3'UTR region of *lin-14* and negatively regulates its expression. Another miRNA, let-7 which is a heterochronic gene of *C. elegans* was discovered in 2000, seven years after the discovery of the first miRNA. It was reported that let-7 is a 21 nt long RNA molecule controlling L4-to-adult transition during the larval development [12]. The discovery that let-7 miRNA is conserved across many species initiated a wide search for microRNAs in various organisms. Presently thousands of miRNAs have been identified in humans and other species. Numerous miRNA prediction tools, such as miRbase database, have facilitated the prediction of miRNA and their target genes in different organisms.

2.1. Mechanism of miRNA-mediated gene silencing

2.1.1. Initiation

Gene silencing by miRNA may occur either by mRNA degradation or preventing mRNA translation. RNAi machinery can be triggered by RNA molecule generated from a variety of sources. Primary miRNA or pri-miRNA, which serves as a source of endogenous dsRNA, folds on itself and forms a double-stranded stem-loop structure. Some microRNA precursors located in the intergenic regions are transcribed by the gene promoters, while others clustered in the polycistronic transcripts are driven by their own promoters [13]. miRNA precursors are mainly transcribed by RNA polymerase II and infrequently by RNA polymerase III [14, 15]. The conversion of nascent pre-miRNA into mature miRNA takes place in two steps. First, the primary miRNA transcripts (pri-miRNA) are processed to form ~70nt pre-miRNA. Then the pre-miRNA is further chopped to generate 21– 25 nucleotide mature miRNAs [16] (Fig. 2). The two chronological steps of miRNA maturation are executed by two dsRNA-specific endonucleases Drosha and Dicer. These enzymes cleave dsRNA in a specific manner and thus generating 3' overhang nucleotides and 5' phosphate groups. Drosha in association with another protein "Pasha" (DGCR8), recognizes the pri-miRNA and excises its stem-loop structure inside the nucleus. A Ran-GTP dependent transporter, exportin-5 transports the pre-miRNAs from the nucleus into the cytoplasm [17]. Once the pre-miRNA enters the cytoplasm, the Dicer will trim off its loop and process it into a mature microRNA [18]. The processing of exogenously introduced dsRNA involves Dicer but not Drosha and Pasha. Dicer is endoribonuclease from the RNase III family which cuts dsRNA and pre-microRNA (pre-miRNA) into double-stranded siRNA and miRNA, respectively. It is a complex protein that contains a dsRNA binding domain, a helicase domain, two RNase III domains , and a Piwi/Argonaute domain [19]. In humans, the protein is encoded by the *DICER1* gene containing helicase and PAZ (Piwi/Argonaute/Zwille) domains [20]. The PAZ domain binds to the 3' overhang of dsRNA and the RNaseIII catalytic domains initiate the cleavage of the RNA strands [21]. Dicer associates with the existing terminus of dsRNA and cleaves ~21 nucleotides from the end thus forming a fresh end with two 3' overhangs. Due to the continuous terminal cleaving of dsRNA, a pool of ~21-nt long small RNA with two 3'overhangs nucleotides are generated.

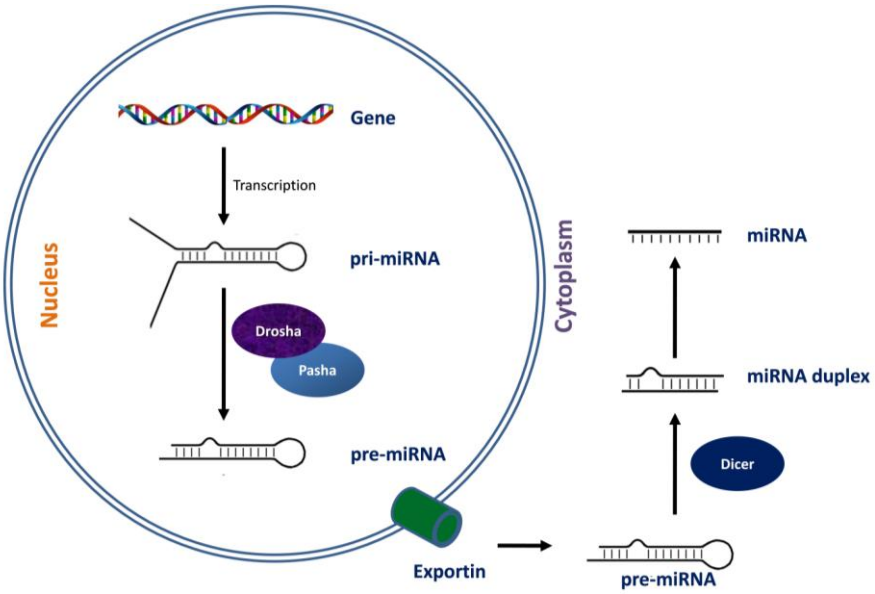

Fig. 2: Representative scheme of biogenesis of microRNAs. Inside the nucleus, the pri-miRNA transcripts are processed by Drosha into pre-miRNAs. Exportin-5 transports pre-miRNA into the cytoplasm where they are processed by Dicer. One of the strands of the miRNA duplex is loaded into RISC and guides the complex towards the target mRNA.

2.1.2. Assembly of RNA-induced silencing complex (RISC)

The miRNAs, generated due to the activity of dicer, assemble with the ribonucleoprotein complex called as RNA-induced silencing complex (RISC). RISC incorporates one strand of a single-stranded RNA (ssRNA) like miRNA or double stranded siRNA and uses it as a guide to suppress the expression of a specific mRNA [22]. RISC complex is composed of different proteins and the most conserved members are Argonaute proteins, which play a central role in the activity of this complex. Argonaute proteins are involved in degradation of the target mRNA strand complementary to the bound siRNA [23]. Structural analysis demonstrated that PIWI domain is a possible functional domain of Argonaute protein as it possesses the RNase H activity [24]. The numbers of Ago proteins varies from organism to organism and ranges from one in *S.pombe* to over twenty in *C.elegans* [25]. There are eight Ago proteins present in humans but only Ago-2 has the active catalytic domain involved in the RNA cleavage function [26].

2.1.3. Effector stage

Most of the data suggest that effector stage of miRNA-mediated post-transcriptional gene silencing takes place in the cytoplasm and P-bodies. Double-stranded microRNA is a transient imperfect duplex molecule consisting of a passenger strand and a mature guide. The RNA duplex is unwound and the guide strand is incorporated into the RISC protein complex to direct the complex towards target mRNA. In *Drosophila*, the RISC assembly model involves ATP-dependent unwinding of RNA duplexes thus enabling the guide strand to load into Ago2 of the RISC complex leading to its activation. The process of miRNA duplex unwinding is followed by subsequent degradation of the passenger strand by Ago2 [27]. Whether passenger strand degradation occurs prior to RISC loading is not clear. The key feature of binding of miRNA and target mRNA involves the Watson-Crick base pairing between the guide strand and the 3'UTR of the target. Most plant miRNAs bind to the coding sequence of target mRNAs with a near-perfect complementarity. On the other hand, animal miRNAs have been observed to bind the target mRNA with lots of mismatches and bulges. The degree of miRNA-mRNA complementarity is a key determinant of the gene regulation. The perfect complementarity allows Ago-catalyzed degradation of the mRNA strand, as the central mismatches promote repression of mRNA translation rather than mRNA cleavage [1]. It has been observed that miRNA-directed mRNA degradation is catalyzed by Ago2 when the miRNA and target are extensively base-paired in the seed region and 10-11 bases of the guide strand [26].

In addition to the slicer activity, miRNA can accelerate the target mRNA deadenylation and decapping thus affecting the translation initiation and transcript stability [28]. Translation is also indirectly regulated by spatial separation of miRNA-targeted mRNA complex from translational machinery into the cytoplasmic P-bodies, also known as processing bodies [29]. The function of P-bodies is very crucial for the miRNA activity as the inhibition of P-body formation by depletion of GW182, which is a major component of P-bodies, impairs miRNA function [30, 31]. Active miRNA-RISC complex directs the mRNA awaiting translation or being translated to the P-body, where the degree of miRNA-target complementarity will determine the slicer-dependent or slicer-independent gene silencing.

2.2. Functions of micro RNAs

The key function of miRNAs is to inhibit the protein synthesis either by inhibition of translation or by mRNA degradation. A study based on ribosome profiling to study an

overall effect on protein production as well as mRNA expression levels revealed that mRNA destabilization is a predominant miRNA silencing mechanism rather that inhibition of translation [32]. Not only mRNAs repression, miRNAs have also been discovered to activate translation of targeted mRNAs [33]. It was clearly demonstrated that under the serum starvation conditions, TNFα AU-rich elements recruit miR369-3 to mediate translational upregulation. On the other hand, in the case of synchronized proliferating cells, miR369-3 caused repression of translation. Similarly, during the cell cycle arrest let-7 microRNA induces translation whereas it represses translation in case of proliferating cells. Therefore, miRNAs can switch between translation activation and repression depending on the phase of cell cycle. Many miRNA have been functionally characterized in both animals and plants and have been demonstrated to be involved in various functions. Some of the examples are described below.

2.2.1. miRNAs in Animals

MicroRNAs play an important role in the regulation of biological functions in animals and regulate the expression of genes at various stages of development. In the case of animals, miRNAs exhibit a limited complementarity with their target mRNAs, but still exhibit a firm control over most of the physiological processes. They have been reported to repress the initiation step of the translation process, which is followed by mRNA degradation [34]. Loss-of-function mutants of the first two identified miRNAs in *C. elegans*, lin-4 and let-7 showed defects in larvae development [11, 12]. lin-4 mainly regulates the early development in *C. elegans*, whereas let-7 is involved in late developmental processes. In *Drosophila melanogaster*, many functional miRNA have been discovered. Overexpression of bantam, a *Drosophila* miRNA, has been demonstrated to have antiapoptotic and pro-proliferative activities [35]. In mammals, a muscle-specific miRNA, miR-1, targets HAND2 (heart and neural crest derivatives-expressed protein 2) and results in premature differentiation and muscle degeneration of cardiomyocytes [36]. Certain miRNAs like miR-375 control various physiological processes of an organism. miR-375 inhibits glucose-induced insulin secretion by regulating its target gene Myotrophin, suggesting that miR-375 is an inhibitor of glucose-stimulated insulin secretion [37]. On the other hand, miR-375 is also highly expressed in the pituitary gland of zebrafish, indicating its possible role in the secretion of hormones. Several of the known miRNA in animals and their functions have been listed in Table 1.

Several miRNAs have been demonstrated to have essential roles in normal lung development and different lung diseases [38, 39]. The lung diseases that have been associated with different miRNAs include Asthma, chronic obstructive pulmonary disease (COPD), Cystic fibriosis and lung cancer [39, 40].

2.2.2. miRNAs in plants

Like animals, miRNAs play a critical role in the regulation of growth and development of plants. However unlike animal miRNAs, plant miRNAs show high complementarity with the target mRNAs, which makes prediction and identification of miRNAs easy. In plants, miRNA mainly targets transcription factors and F-box proteins, which form the major plant developmental network [41]. The significance of miRNAs in plant growth and development was discovered from the analysis of mutants impaired in small RNA biogenesis. These mutants show a number of developmental defects including the altered growth patterns. Accumulation of target gene expression was demonstrated in the absence of miRNA activity. miR-165/166 regulates the expression of prohibitin (PHB) in *Arabidopsis* and RLD1 in maize. Mutations in the miR-165/166 complementary site in RLD1 causes an accumulation of RLD1-mRNA, suggesting the role of miRNAs in the regulation of genes involved in the development of maize [42]. In *Arabidopsis*, APETALA2 (AP2) regulates the developmental timing of flowers. AP2 gene expression, in turn, is regulated by miR-172 microRNA. The overexpression of miR-172 causes the loss-of-function of AP2 thus showing floral developmental defects such as the absence of petals and transformation of sepals into carpels [43]. Floral initiation, as well as floral development, is controlled by the plant hormone gibberellin (GA). GAMYB mediates gibberellic acid-dependent pathway in plants and regulates GA-activated genes. GA regulates miR-159 activity and miR-159 directs the cleavage of mRNA encoding GAMYB-related proteins like MYB33 and MYB65 [44]. Overexpression of miR-159 causes the late flowering phenotype, whereas plants expressing the miRNA-resistant version of MYB33 leads to developmental defects like hyponastic leaves [45, 46]. Some of the known plant microRNA and their functions have been listed in Table 2. Genome wide screening of rice led to discovery of different miRNAs including osmiR397 in rice seeds [47]. It was demonstrated that overexpression of the two forms of this miRNA lead to 7.4 and 13.4% increase in 1,000-grain weight, along with the increase in grain size [48].

Table 1: MicroRNAs and their functions in animals.

miRNA	Target gene	Function	Organism	Reference
lin-4	lin-14, lin-28	Larval development	*C. elegans*	[11]
let-7	lin-41	Larval developmental timing	*C. elegans*	[12]
bantam	HID	Proliferative and antiapoptotic	*D. melanogaster*	[58]
miR-1	HAND 2	Cardiomyocyte differentiation	*Mus musculus*	[36]
miR-7	Notch targets	Notch signaling	*D. melanogaster*	[59]
miR-375	Myotrophin	Insulin secretions	*M. musculus*	[37]
miR-32	Retrovirus PFV1	Antiviral defense	*H.sapiens*	[60]
miR-146	c-Myc, ROCK1	Immune system	*H.sapiens*	[61]
miR-223	NFI-A	Granulocytic maturation	*H.sapiens*	[62]

Table 2: MicroRNAs and their functions in plants.

miRNA	Target gene	Function	Organism	Reference
miR156	SPL	Developmental transition time	*A. thaliana*	[63]
miR160	ARF10	Signal transduction	*A. thaliana*	[64]
miR164	NAC-TF	Root and shoot development	*A. thaliana*	[65]
miR157	SPL	Developmental timing	*G. hirsutum*	[66]
miR172	AP2	Floral development	*O. sativa*	[43]
miR319	TCP	Leaf development	*A. thaliana*	[67]
miR390	TAS3	Auxin response	*Z. mays*	[68]
miR393	TIR1	Hormone signaling	*A. thaliana*	[41]
miR394	F-Box	Hormone signaling	*A. thaliana*	[69]
miR399	At2g33770	Ubiquitin conjugation	*A. thaliana*	[70]

2.3. Role of miRNAs in diseases

MicroRNAs represent the critical regulators of gene expression in addition to the transcriptional control. miRNAs regulate the various phases of cell development and the dysregulation of miRNA function can lead to various disorders. Some of the common examples of such disorders include hereditary progressive hearing loss in miR-96 [49]. The first disease to be associated with miRNA dysregulation in humans was chronic lymphocytic leukemia [50]. One of the major underlying causes of cancer is DNA damage and lack of repair system. If a cell is deficient in DNA damage repair, the mutations accumulate and cause epigenetic alterations. Such accumulated mutations and epigenetic alterations can lead to cancer. Altered expression of microRNAs leading to DNA repair deficiencies is often associated with cancers. Almost 15% of deficiencies in MLH1 (a DNA repair protein) in sporadic colon cancers are caused by the overexpression of the microRNA miR-155, which represses MLH1 protein expression

MicroRNA expression profiling studies have demonstrated that the expression profile of specific miRNAs alter during heart diseases in humans, therefore, indicating their role in cardiomyopathy [51]. MicroRNA-712 is a potential biomarker of atherosclerosis, a cardiovascular disease associated with lipid retention and inflammation in arterial wall [52]. miRNAs play a critical role in the differentiation of stem cell progenitors into the adipocytes [53]. Decreased expression of miR-155, miR-121 and miR-122 has been observed during the adipogenic differentiation, suggesting that they act as negative regulators of differentiation. MicroRNAs have been linked to various growth and developmental defects in humans. Deletion of the miR-17~92 cluster has been associated with skeletal and growth defects in humans [54]. miRNAs also regulate the development of nervous system and neural miRNAs like miR-124, miR-132, miR-134 and miR138 have been shown to be involved in the synaptic development and synapse maturation [55, 56]. Various other nervous disorders like bipolar disorder, schizophrenia, and anxiety disorders have also been associated with miRNA expression [57].

3. CONCLUSIONS

After their discovery in 1993, miRNAs became an exciting area of research to elucidate their mechanism of action and function in various organisms. Since the discovery of miRNAs, there was a huge shift of research from coding to non-coding DNA. MicroRNAs are highly conserved non-coding RNA molecules which regulate the expression of different genes at various levels. The regulatory functions of these small RNA molecules are carried out mainly through the RNA-induced silencing complex. In both plants and animals, numerous miRNAs and their targets have been discovered and many vital functions in growth and development have been attributed to them. There are evidences that miRNA dysregulation is associated with various diseases notably cancer and heart diseases. MiRNAs are currently under extensive research for future applications in cancer diagnosis, prognosis and treatment.

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