Chapter 2

MicroRNA Expression: Protein Participants in MicroRNA Regulation

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Abstract

MiRNAs are ~20 nt small RNAs that regulate networks of proteins using a seed region of nucleotides 2–8 to complement the 3′ UTR of target mRNAs. The biogenesis and function of miRNAs as translational repressors is facilitated by protein counterparts that process primary and precursor miRNAs to maturity (Drosha/DCGR8 and Dicer/TRBP respectively) and incorporate miRNAs into the protein complex RISC to recognize and repress target mRNAs (RISC proteins: Ago/TRBP1/TRBP2/DICER). Similarly, siRNAs through comparable mechanisms are loaded into the protein complex RITS to heterochromatin formation of DNA and suppress transcription of particular genes. MiRNAs are also regulated themselves through many different pathways including transcriptional regulation, post-transcriptional RNA editing, and RNA tailing. Dysregulation of miRNAs and the protein participants that mature them are implicated in the development of a number of diseases, tumorigenesis, and arrested development of embryonic cells. In this chapter, we will explore the biosynthesis, function, and regulation of miRNAs.

Key words Dicer, Drosha, miRNA, mRNA, Protein, RISC, Regulation

1 MicroRNA Production and Activity

MicroRNAs are small noncoding RNAs around 22 nucleotides in length that are involved in the regulation of mRNAs in the cytoplasm via inducing translational repression or message degradation [1]. MiRNAs constitute a broad regulatory network with one miRNA potentially regulating dozens of distinct mRNAs. These regulatory networks control levels of specific proteins and other RNAs like long noncoding RNAs (lncRNAs) in cells. MiRNA misregulation is implicated in cancer, a myriad of other illnesses, and abnormal development [2–4]. The first miRNA was described in 1993 and was initially thought to be a novel molecular species unique to *Caenorhabditis elegans* [5]. In 2001, however, nearly 10 years after their initial discovery, miRNAs were found to occur in several different species including humans [6]. Since that time, novel miRNA discovery has proceeded at a marked rate, and by

2015, MiRBase.org [7] has detailed significant evidence supporting the expressions of over 2500 unique human miRNAs and well over 28,000 unique miRNAs across species.

1.1 MiRNA Processing

MiRNAs are transcribed from the genome by RNA Polymerase II (Pol II) or Pol III [1]. Transcription results in an initial transcript (called a primary miRNA or pri-miRNA) of variable length containing an unprocessed hairpin [8]. The pri-miRNA is next processed by a ribonuclease protein complex including DROSHA, which targets and cleaves the flanking ends of the hairpin, and DCGR8 that stabilizes the complex on the pri-miRNA. DROSHA processing yields an ~70–100 nt long stem loop called a precursor miRNA or pre-miRNA [9].

Following excision, the pre-miRNA stem loop is transported out of the nucleus and into the cytoplasm via the transport protein exportin-5 using an active transport mechanism with GTP. Once in the cytoplasm, the pre-miRNA is targeted by another ribonuclease, DICER, which cleaves the molecule further by removing the loop portion of the hairpin and leaving an intermediate duplex which consists of the mature miRNA and a semi-complementary sequence referred to as the passenger strand. The intermediate duplex, which is ~22 base pairs in length, is then loaded into an Argonaute (Ago) protein, and the passenger strand discarded [1] (*see* Fig. 1).

1.2 Genomic Loci

MiRNAs can be separated into two broad categories depending on their position in the genome: canonical and noncanonical [1]. Canonical miRNAs are those that are found in intergenic regions and are cleaved by Drosha/DCGR1 to form the precursor miRNA (pre-miRNA) [1, 2]. Noncanonical miRNAs are mitrons or pre-miRNAs that are cleaved from intron sequences using splicing instead of Drosha.

While the evolutionary origin of miRNAs is still largely unknown, significant evidence suggests that miRNAs and their regulatory networks arose from the insertion of transposable elements in the genome [10]. Importantly, the ability of miRNAs to regulate multiple distinct genes may have directly arisen as a consequence of transposons inserting themselves into the UTRs of protein coding genes. Since miRNAs target mRNAs through sequence complementarity, the ability of a miRNA to identify and target a specific mRNA may well be due to a common molecular origin shared by a miRNA locus and its mRNA target sites [10–12] (*see* Fig. 2).

1.3 Translational Repression and Signal Degradation

The mature miRNA in conjunction with the Ago protein is called an RNA-induced silencing complex or RISC. MiRNAs function as protein level regulators by binding target mRNAs and inducing transcriptional repression and in some instances complete signal
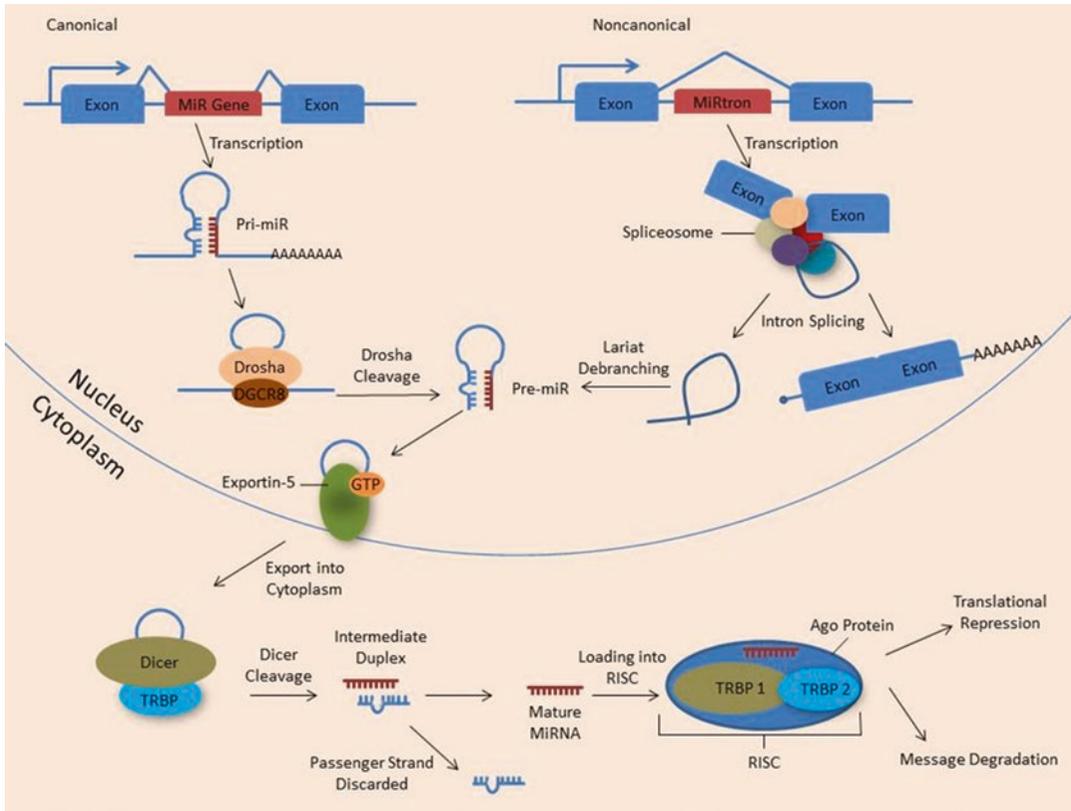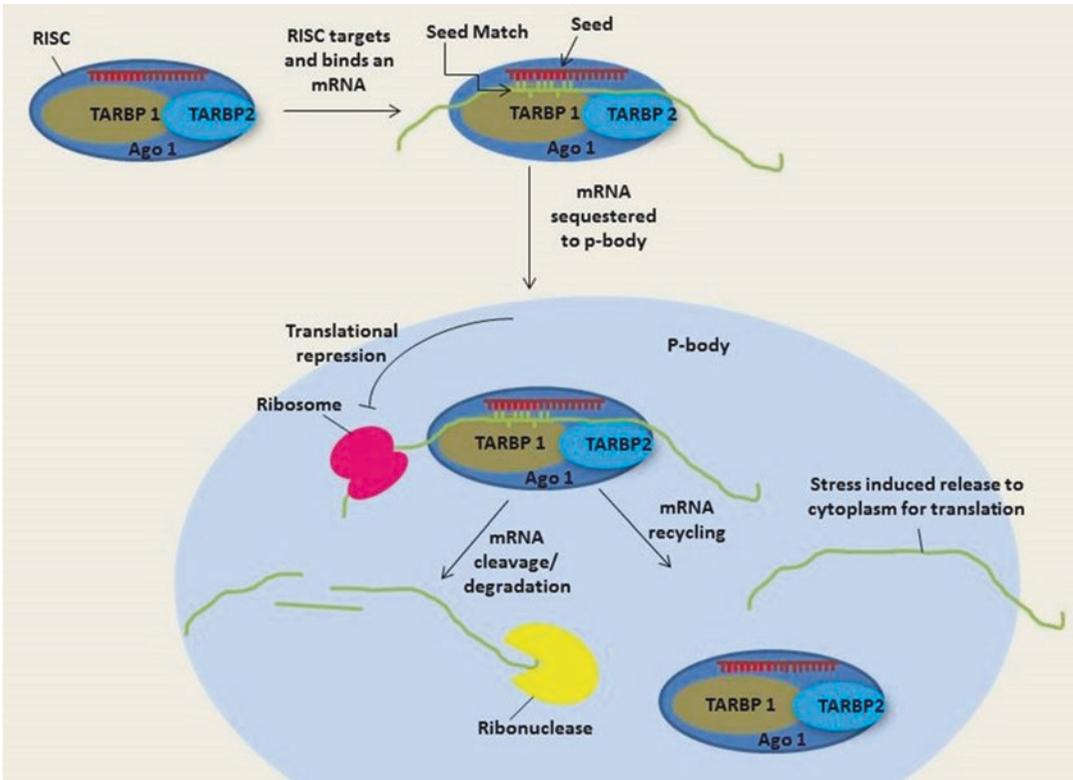
Fig. 1 Canonical and noncanonical miRNA maturation pathways. Cartoon illustrating the transcription of a canonical miRNA, Drosha/DGCR8 processing of the primary hairpin, and the export of the resulting hairpin to the cytoplasm mediated by exportin-5 and GTP. Once in the cytoplasm, the hairpin is recognized and bound by DICER which cleaves the loop off of the stem loop and leaves a double-stranded intermediate duplex. The mature strand bound to the passenger strand is then loaded into an Ago protein complex called RISC. RISC transports the miRNA to a prospective mRNA target which the miRNA recognizes and binds. Once the RISC complex is bound to an mRNA, translation of the mRNA cannot occur and is repressed

degradation. Included in a mature miRNA is a ~7 nt sequence (nts 2–8) called the miRNA seed that perfectly complements a specific region of a mRNA called a seed match usually found in the 3′ UTR. A miRNA seed binds a corresponding seed match in a mRNA, and RISC inhibits its translation. RISC localizes mRNAs that are under transcriptional repression to p-bodies where the mRNA is eventually degraded or released back into the cytoplasm for translation [4]. If the seed is highly complementary to the seed match, the Ago2 protein associated in RISC cleaves the mRNA and results in signal degradation (*see* Fig. 3).

1.4 RISC   The regulation of mRNAs by miRNAs is accomplished by the RNA-induced silencing complex, or RISC. RISC consists of several different proteins, is between 200 and 500 kDa and exhibits

Fig. 2 MiRNA regulatory networks arising from transposable elements. Cartoon depicting transposable line elements being transcribed and inserted into the genome in multiple locations including in the untranslated region of a protein-coding gene. To produce a miRNA hairpin, two line elements are inserted in the genome juxtaposed to each other on different strands. MiRNAs are able to recognize target mRNA seed matches because they each share part of a line element sequence

ribonuclease activity [13]. MRNAs are incorporated into the RISC complex when targeted by miRNAs. AGO proteins in RISC cleave mRNAs that are highly complementary to the incorporated miRNA, whereas mRNAs that are mostly imperfectly bound to the miRNA are silenced by translation inhibition. The best described protein subunits of RISC are the RNases Dicer, Ago 1 and 2. Ago 1 and 2 serve as the central components of RISC and are responsible for translational repression and cleavage/degradation [14]. Also included in RISC are TAR RNA binding proteins, or TARBPs, which comes in two isoforms, TARBP1 and 2. TARBP proteins contain domains that bind double-stranded RNA [15]. TARBP1 functions as a methyltransferase that recruits an Ago protein into RISC [16]. Additionally, TARBP2 loads the miRNA into RISC and exhibits a double-stranded RNA (dsRNA) binding site, which holds the miRNA inside RISC [17, 18].

In addition to loading RNAs into RISC, TRBPs also stabilize Dicer during pre-miRNA processing. After an incorporated miRNA binds to an mRNA, the Ago protein either cleaves the mRNA for degradation, which is usually associated with Ago 2, or the protein

Fig. 3 Translational regulation through seed matching. Cartoon showing the seed region of a mature miRNA incorporated into RISC recognizing and binding to the seed match region of an mRNA. RISC takes the mRNA to the p-body region where it represses translation or cleaves the mRNA for degradation

in conjunction with RISC prevents translation from occurring until the message is degraded or the message is released by RISC through stress-induced pathways and is recycled [19] (*see* Fig. 3).

1.5 SiRNAs and RITS   SiRNAs, or small interfering RNAs, are noncoding RNAs with comparable size and function to miRNAs. SiRNAs differ from miRNAs in several aspects including the pathways from transcription to maturity, and in that while miRNAs generally regulate mRNA networks, siRNAs typically regulate a specific target gene [17]. RITS, or RNA-induced initiation of transcriptional silencing, is a complex of proteins in conjunction with a mature siRNA that inhibits the transcription of specific genes by triggering heterochromatin assembly in centromeric regions.

SiRNA and RITS complexes have been experimentally characterized in fission yeast. In fission yeast the RITS complex consists of Ago1; Chp1, which is a hetero-chromatin-associated protein; and Tas3, a novel protein that is necessary for H3-K9 methylation [20]. This protein complex, in conjunction with a mature siRNA, targets specific regions of DNA and silences them using methylation and heterochromatin biogenesis [21]. Upon being loaded
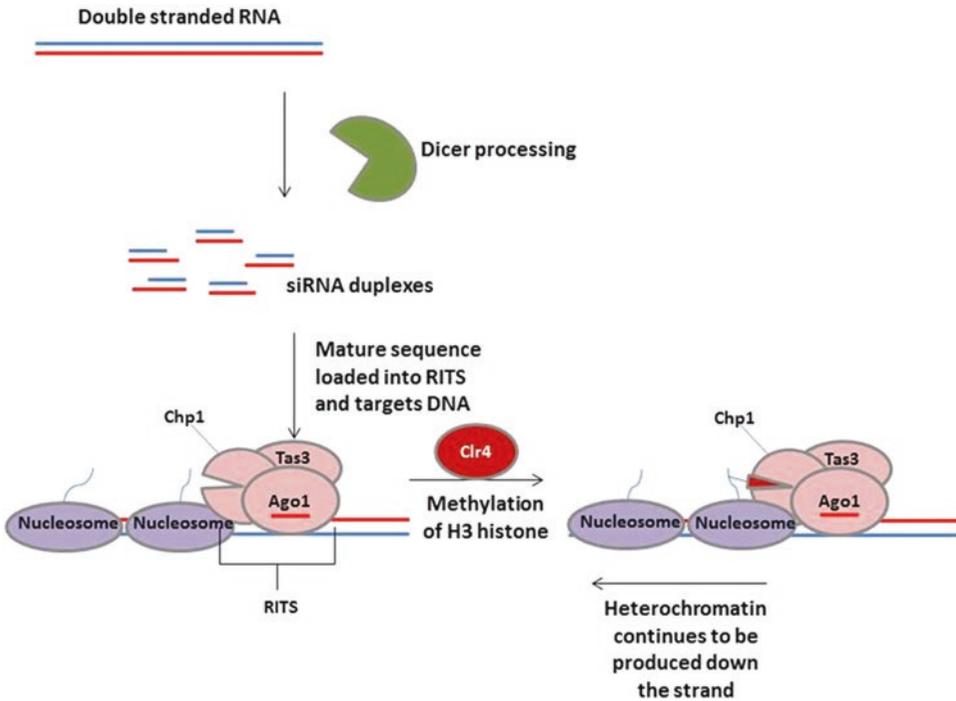
Fig. 4 DNA methylation facilitated by RITS and siRNA is a cartoon depicting dsRNA or double-stranded RNA being cleaved by Dicer and matured into an siRNA or small interfering RNA. RITS is composed of at least three protein components including Ago1, Chp1, and Tas3. The RITS protein complex, in conjunction with the siRNA, binds to a centromeric region of DNA specified by the siRNA and enables methylation of the H3 histone by Clr4, an H3 methyltransferase

into Ago1, a siRNA will bind a complementary centromeric region of DNA in complex with the RITS proteins. Then K9 methylation of the H3 histone is initiated by Clr4 protein, a histone H3 methyltransferase [22]. In response to this methylation, targeted DNA coils tightly around its associated histones thereby preventing transcription of the region (*see* Fig. 4).

2   MiRNA Regulation

Importantly, miRNAs are themselves regulated through several different mechanisms such as transcriptional regulations, single nucleotide polymorphisms (SNPs), RNA editing, miRNA tailing, and miRNA degradation [23]. MiRNA regulation can alter miRNA expressions and protein targets, affect miRNA dosing and miRNA proliferation, and induce miRNA degradation.

2.1   Regulation of MicroRNA Transcription

Transcriptional regulation of miRNAs is perhaps the most well-understood mechanism by which to regulate miRNA biogenesis [24]. MiRNA locus methylation is the untemplated addition of

methyl groups to a region of DNA in order to encourage tight heterochromatin folding of DNA that prevents transcription. MiRNAs can be down regulated when regions in the DNA that code for the miRNA and regions that allow the miRNA to be transcribed such as a promoter or transcription factor are silenced by gene inaccessibility [25]. Hypomethylation plays a role in up-regulated levels of miRNAs in the cell.

Regions that are normally inaccessible to transcription can be "opened up" by mechanisms that are as yet not fully understood. Hepatocellular carcinoma (HCC) is associated with the dysregulation of miRNAs by hypermethylating regions of the DNA which include tumor-suppressing miRNAs and hypomethylating regions that are normally constitutively transcribed and causes the up-regulation of miRNAs that can promote tumor growth [26].

Additionally, transcription can also be affected by levels of particular proteins (typically transcription factors) in the cell. For instance, mir-145 has been shown to produce an apoptotic effect in cells following an activation of TP53, a tumor suppressor. The production of TP53 also stimulates the transcription of miRNA-145 creating an apoptosis-promoting loop. Conversely, miRNA-145 under-expression is observed in a variety of cancers including breast, colon, and lung cancers [27].

Further, transcriptional regulation of miRNAs has been shown to play a major role in embryonic stem cell (ES cell) differentiation [28]. Four transcription factors have been implicated in regulating miRNA levels in the cell to induce differentiation in mice: Oct4, Sox2, Nanog, and Tcf3. Using CHIP-seq data and intensive miRNA promoter mapping, Marson et al. were able to show that the transcription factors bind to promoter regions that are responsible for the transcription of at least 81 miRNA genes and inhibit particular miRNAs from being transcribed [29]. This process results in the shift of ES cells from pluripotency to specificity [30]. The transcription factors bind to the promoter regions of miRNA genes and prevent the gene from being polymerized which in turn results in increased expression of proteins that the targeted miRNA regulates.

2.2 Single Nucleotide Polymorphisms

Single nucleotide polymorphisms or SNPs are areas in the genome where a single nucleotide can differ between individuals due to typically benign mutations in the DNA sequence [31]. That said, SNPs can drastically alter miRNA activity in the cell. A SNP in the seed sequence of a miRNA can significantly alter its targets and allow a certain protein to go uninhibited while another protein becomes more stringently regulated. This can cause issues when oncogenes, for example, are no longer being targeted to be a particular miRNA, or if tumor suppressor proteins are severely repressed following the introduction of an SNP in a miRNA that does not normally regulate the tumor suppressor gene [23]. SNPs

can also lead to miRNA regulation when it is not localized in the seed region. SNPs in the passenger strand or pri-miRNA stem loop can interfere with Drosha and DICER processing and inhibit the production of a mature miRNA [32].

2.3   RNA Editing

MiRNAs can also be regulated through RNA editing. A protein called ADAR1 can convert adenosine molecules into inosines that preferentially form base pairs with cytosines [33]. RNA editing may affect pri-miRNAs, pre-miRNAs, and mature miRNA sequences, and like SNPs, these edits can alter gene targets or decrease the affinity for Drosha or DICER affecting miRNA processing [34].

Importantly, it is estimated that 16% of human pri-miRNAs are edited by ADAR1 [23], suggesting that ADAR editing may well provide a largely underappreciated layer of complexity in miRNA biogenesis. Although miRNA editing can allow the production of several unique miRNAs with differing targets to be produced from a single genomic locus, the effects of miRNA editing remain largely unexplored.

2.4   MiRNA Tailing

RNA tailing is the post-transcriptional addition of nucleotides to the 3′ end of RNA. Uridylation mainly occurs in pre- and pri-miRNAs. For example, during embryonic stages, members of the let-7 family are suppressed after transcription by LIN28A and its paralogue LIN28B. These proteins bind to the terminal loop of pri-let7 and pre-let-7 respectively, and prevent Drosha and Dicer processing. LIN28 proteins then employ terminal uridylyl transferases TUT4 and TUT7 to signal pre-let-7 for decay by inducing oliguridylation [35, 36]. Next, DIS3L2 exonuclease targets the oligo-U tail and degrades the miRNA. Conversely, when LIN28 is down regulated in cells, TUT7, TUT4, and TUT2 stimulate monouridylation of pre-let-7, which increases let-7 proliferation [37].

Another type of RNA tailing is adenylation, which primarily occurs in mature miRNAs. Adenylation can result in either miRNA stabilization or miRNA decay [38]. As examples, miRNA-122, a hepatic miRNA, is frequently stabilized by adenylation, whereas poxvirus polyadenylation polymerase targets host miRNAs and adenylates them causing their degradation. It remains unclear what causes the difference in response following miRNA polyadenylation [39].

2.5   MiRNA Degradation

Mature miRNA degradation has been observed in several different systems. Though it is unclear how nucleases specify targets, numerous nucleases are suspected of actively degrading miRNAs in humans [18], *C. elegans* [40], and mice [41]. The first reported instance in which miRNAs were rapidly degraded was observed in *Arabidopsis thaliana*, in which mature miRNAs were cleaved and removed by an association of 3′–5′ exonucleases called small-RNA-degrading nuclease [42].

Similarly, it has been shown that viruses destabilize specific miRNAs by using their own RNA that contains a perfectly complementary sequence [43]. For example, T cells that are infected with herpes virus experience a rapid decrease in miRNA-27 due to viral noncoding RNA specifically binding and destabilizing miRNA-27 [44]. Also, mouse cytomegalovirus contains RNA that specifically binds miRNA-27 and facilitates its degradation [45].

3 Concluding Remarks

Though miRNAs genes were almost entirely overlooked until the turn of the millennium, miRNAs have now been shown to play an integral part in the post-transcriptional regulation of many (if not a majority of) protein genes. What is more, our understanding of miRNAs and their functions as regulators continues to broaden with significant new insights continuing to be described. For example, miRNAs were recently shown to target not only mRNAs, but also other noncoding RNAs such as long noncoding RNAs or lncRNAs. Linc-MD1 is a lncRNA that is expressed during myoblast differentiation in muscle cells. MiRNA-133 and -135 down regulate two transcription factors that stimulate muscle-specific gene expression, MAML1 and MEF2C respectively. Linc-MD1 competes with these transcription factors to bind the miRNAs and allow the transcription factors to initiate cell differentiation [46]. As another example, other small RNAs have now been shown to behave like mature miRNAs. For example, many snoRNAs, or small nucleolar RNAs, classically thought to simply chemically modify other RNAs, have now been reported to undergo alternative processing and behave like mature miRNAs. In a recent study, specific snoRNAs were found to be processed into miRNA-like fragments and direct translational repression of target genes [47].

In conclusion, while we have learned a lot about miRNA production and regulation in a very short time, our understanding of these molecules is still in its infancy, and exciting new revelations undoubtedly await.

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