

Chapter 2

Biogenesis and Physiology of MicroRNAs

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Abstract MicroRNAs (miRNAs) are small noncoding RNAs 17–25 nucleotides long that control gene expression by promoting degradation or repressing translation of target mRNAs. Since each miRNA regulates the expression of hundreds of target mRNAs, miRNAs can be seen as master-coordinators, efficiently regulating fundamental cellular processes such as proliferation, apoptosis, and development. MiRNAs are synthesized in the cell through a multistep coordinated process that starts in the nucleus and proceeds to the cytoplasm culminating with the production of the biological active form, the miRNA. This maturation process consists of a series of biochemical steps that convert the primary miRNA transcript into an intermediate precursor miRNA hairpin and culminates with the formation of the mature miRNA. The spatiotemporal control of miRNA abundance is made possible, in part, by the regulation of its biosynthesis pathway, where alterations can lead to global miRNA deregulation. Since miRNAs are involved in a broad range of developmental and physiological processes their deregulation appears to play a fundamental role in the onset, progression, and dissemination of many cancers as well as in many other human diseases.

Keywords miRNAs • Biogenesis • Maturation • Drosha • Dicer • Cropping • Dicing • Slicing

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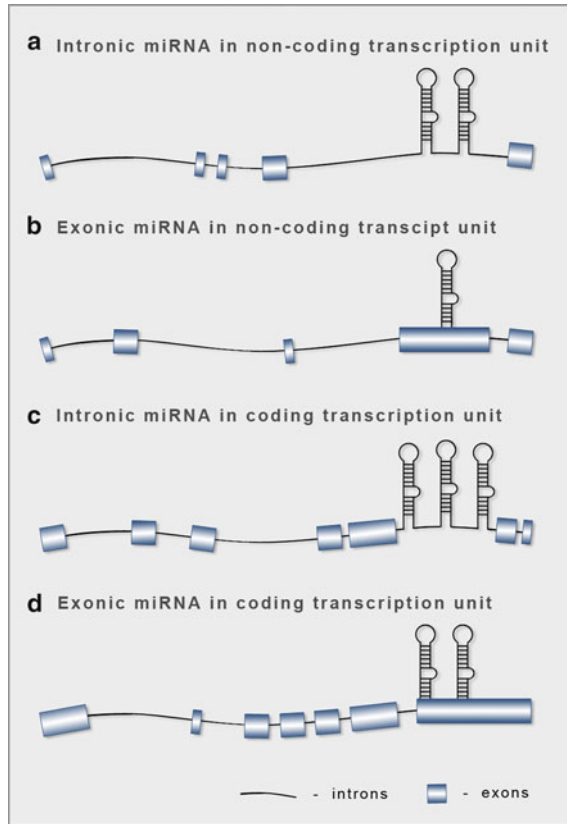
2.1 MicroRNAs

2.1.1 Definition and Genomic Organization

MicroRNAs (miRNAs) are a class of small (17–25 nucleotide; nt), single-stranded noncoding RNAs that control gene expression in animals, plants, and unicellular eukaryotes that have emerged as key posttranscriptional regulators of gene expression [1–3]. The first studied miRNA gene was *lin-4*, which was identified in *C. elegans* in 1993 by Victor Ambros and colleagues during genetic studies of defects in larval development [4]. Seven years later, Reinhart et al. discovered a second 22-nucleotide small RNA, *let-7*, a gene that was also involved in *C. elegans* developmental timing [5]. These reports found that the 22-nt *lin-4* and 21-nt *let-7* are both translational repressors of mRNAs [4, 5]. These small molecules gained the attention of the scientific community for two main reasons: homologs of the *let-7* gene were identified in other animals including humans that suggested an important and fundamental biological role for this small RNA; likewise RNA interference (RNAi) was discovered at that time, and it became clear that both pathways were linked and shared common components. Within the following year, more than 100 additional small RNAs were identified in worms, *Drosophila*, and in humans [6]. Currently, more than 18,226 miRNAs from 50 organisms are registered in the miRNA database (<http://www.mirbase.org/>). These small RNAs have emerged as key posttranscriptional regulators of gene expression controlling about ~60 % of all protein-coding genes in mammals. Thus, miRNAs participate in the regulation of almost every cellular event such as development, differentiation, proliferation, and apoptosis.

The genomic distribution of miRNA genes is characterized by the presence of families of several identical or closely related mature miRNAs, encoded within the same genomic cluster. Therefore it is expected that a certain degree of redundancy will exist among miRNAs [7, 8]. In humans approximately 50 % of known human miRNAs are found in clusters and they are transcribed as polycistronic primary transcripts [6, 9]. There are usually two or three genes per cluster and the largest cluster, at 13q31, is composed of seven [10, 11]. This genomic organization confers simultaneous expression of similar miRNAs, possibly leading to a synergistic effect in their ultimate function. Moreover, a significant portion of miRNAs are located in the intronic region of protein-coding and/or -noncoding transcription units, whereas a minor subset of miRNAs are mapped to repetitive sequences such as long interspersed nuclear elements [12, 13]. There are four groups of miRNA genes according to their genomic location: intronic miRNA in noncoding transcription units; exonic miRNA in noncoding transcription units; intronic miRNA in protein-coding transcript units or exonic miRNAs in protein-coding transcripts (Fig. 2.1). About one-third of miRNA genes are located in the introns of protein-coding genes; these are found on the sense strand, implying some linkage of miRNA and host mRNA transcription [12, 14]. Human miRNAs are located in all chromosomes except Y chromosome and they are nonrandomly distributed in the human genome.

Fig. 2.1 MicroRNA genomic location and gene structure. MicroRNAs can be categorized into four groups according to their genomic location: (a) MiRNAs located in introns of noncoding transcripts, (b) miRNAs located in exons of noncoding transcripts, (c) miRNAs in introns of protein-coding transcripts, and (d) miRNAs in exons of protein-coding transcripts



2.2 Biogenesis and Maturation of MicroRNAs

The biosynthesis of miRNAs is a tightly regulated multistep process that starts in the nucleus of the cell, following transcription, and continues through the cytoplasm where finally the mature miRNA molecule exerts its main function (Fig. 2.2). Each one of the multiple steps that compose miRNA biosynthesis seems to be remarkably well coordinated. Drosha initiates the processing by specific cropping of the stem-loop precursor in the nucleus [15]. The resulting structure, precursor miRNA (pre-miRNA), seems to be a signature motif for all dsRNAs that are involved in small-RNA pathways. Exportin-5 recognizes this signature motif and exports pre-miRNAs to the cytoplasm through nuclear pores on a GTP–GDP gradient [16, 17]. Following export, pre-miRNA is handed over to another RNase III enzyme, Dicer, that dices the pre-miRNA into a miRNA duplex that is further unwinded giving rise to the mature functional miRNA molecule.

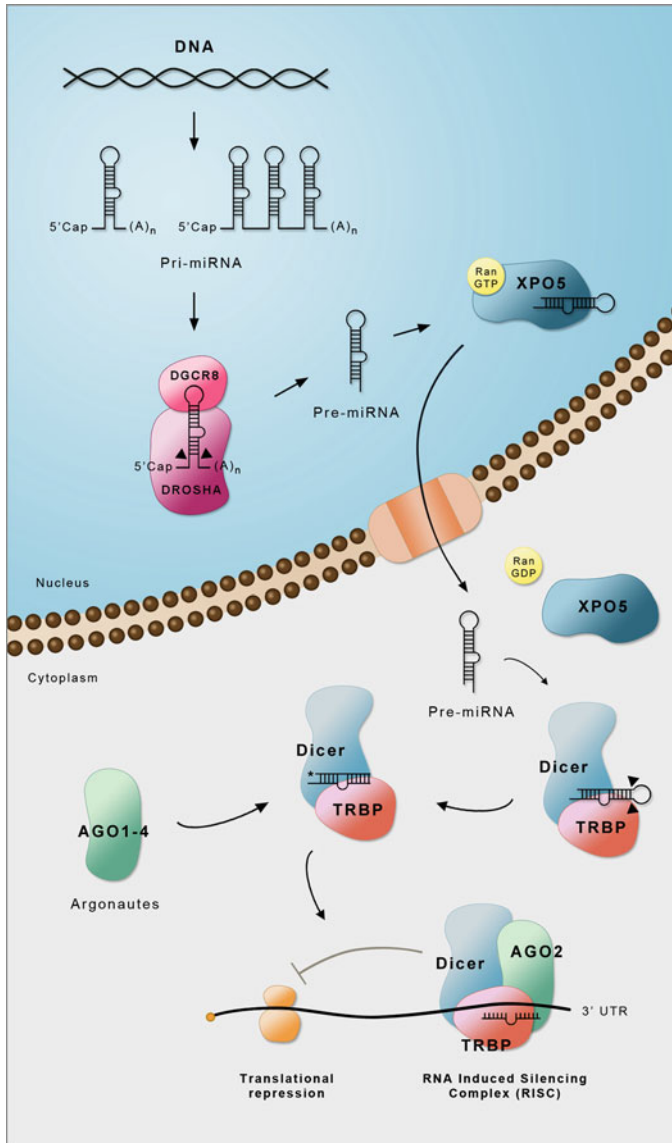


Fig. 2.2 MicroRNA biogenesis pathway. Long primary transcripts (pri-miRNAs) containing one or several miRNAs are transcribed by RNA polymerase II and cleaved by the microprocessor complex, containing at least Drosha (RNAase III endonuclease) and DGCR8 in humans (a double-stranded RNA-binding protein). This complex recognizes the double-stranded RNA structure of the pri-miRNA and specifically cleaves at the base of the stem-loop, hence releasing a 60- to 70-nucleotide precursor (pre)-miRNA. This pre-miRNA is then exported through the exportin-5 pathway into the cytoplasm where it is further processed into a mature miR/miR* duplex by DICER1, a second RNase III endonuclease together with its catalytic partner TAR-binding protein (TRBP). The miR/miR* duplex is then loaded into a multicomponent complex, the RNA-induced silencing complex (RISC), constituted of at least TRBP, DICER1, and one Argonaute (Ago2 in human). The miR serves as a guide for target recognition while the miR* passenger strand is cleaved by Ago2. Most of animal miRNAs harbor an imperfect homology with their targets and, therefore, inhibit translation by an RISC-dependent mechanism

2.2.1 *Canonical Versus Noncanonical Processing of MicroRNAs*

2.2.1.1 **MiRNA Transcription and Drosha “Cropping”**

The biogenesis of a miRNA begins with the synthesis of a long transcript known as primary miRNA (pri-miRNA). The promoter region of autonomously expressed miRNA genes is highly similar to that of protein-coding genes [18, 19]. pri-miRNAs are typically (although not exclusively) transcribed by RNA polymerase II, which generates the pri-miRNA that consists of one or more hairpin structures, each composed of a stem and a terminal loop. pri-miRNAs are structurally analogous to mRNAs; they are 5'-capped and spliced and bear a 3' poly-A tail, and they often can produce more than one functional miRNA [20–23]. However, other pathways generate a minor set of miRNAs, especially from genomic repeats (i.e., Alu repeats) whose transcription is carried out by RNA polymerase III [24].

In the canonical miRNA biogenesis pathway the first step of miRNA maturation starts in the nucleus where the pri-miRNA is “cropped” into a ~70 nt hairpin-structured pre-miRNA. This phenomenon is catalyzed by a multiprotein complex called the microprocessor. The core components of this complex are Drosha, an RNase III enzyme, together with its interacting partner DGCR8 (DiGeorge syndrome critical region gene 8), a double-stranded RNA-binding domain (dsRBD) protein [15, 25, 26]. The DGCR8 protein recognizes the stem and the flanking single-stranded RNA (ssRNA) and serves as a ruler for Drosha to cut the stem approximately 11 nt away from the stem–ssRNA junction releasing the pre-miRNA [27]. The pre-miRNA has a 5' phosphate and a 2 nt 3' overhang, characteristic for RNase III endonuclease products [15, 28]. RNase III enzymes, like Drosha, are a family of double-stranded RNA (dsRNA) ribonucleases that are expressed in all living cells [29]. The microprocessor appears to represent a heterotetramer consisting of two Drosha and two DGCR8 molecules. Importantly, the ~120 kDa DGCR8 protein contains two consensus dsRNA-binding domains; thus DGCR8 may play a critical role in mediating pri-miRNA recognition and/or binding by the microprocessor complex. Moreover DGCR8 gene is located in a region of chromosome 22 that is monoallelically deleted in patients suffering from a relatively common and very complex genetic disease termed DiGeorge syndrome that raises the possibility of the existence of a link between DiGeorge syndrome and global impairment of miRNA processing [30]. Other cofactors play an important role on the action of the microprocessor complex, including the DEAD box RNA helicases p68 (DDX5) and p72 (DDX17), as well as heterogenous ribonucleoproteins (hnRNPs) [26]. All the components of this complex are necessary for pri-miRNA processing in vivo, as reduction on the level of either Drosha or DGCR8 led to the reduction of both pre-miRNAs and mature miRNAs [25, 26]. Drosha cleavage generates a product with a 2 nt 3' overhang that is recognized by exportin-5 (XPO5), which transports the pre-miRNA into the cytoplasm through a Ran-GTP-dependent mechanism [21, 31].

While most miRNAs are generated through the concerted action of the referred enzymes, some animal miRNAs adopt alternative pathways for their maturation. A particular subset of miRNAs known as mirtrons are pre-miRNA-like hairpins that bypass the Drosha requirement step by splicing and debranching of short-hairpin introns [32–34]. Mirtron products appear as pre-miRNA mimics from splicing reaction and enter the canonical biogenesis pathway as XPO5 substrates. Also, some small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), and endogenous short-hairpin RNAs (shRNAs) are also processed into miRNA-like molecules independently of the microprocessor complex [35–38]. In addition terminal hairpins of endogenous siRNA long-stem-loop precursors are another source of miRNAs that do not go through the Drosha processing step [39]. Therefore, despite Drosha processing being the main route by which biosynthesis of miRNAs takes place in the nucleus, some miRNAs have been described to bypass the microprocessor step and enter the biogenesis pathway directly to XPO5.

2.2.1.2 Nuclear Export and Cytoplasmic Processing of MicroRNAs

Following nuclear processing by Drosha the pre-miRNA is recognized by exportin-5 and transits to the cytoplasm to be further processed [16, 17]. Owing to compartmentalization of the processing events, nuclear export of pre-miRNAs is a crucial step in miRNA biogenesis [40, 41]. Although XPO5 was originally known as a minor export factor for tRNAs, the major cargos of XPO5 turned out to be pre-miRNAs. Exportin-5 is a member of the karyopherin family of nucleocytoplasmic transport factors that depend on their cofactor Ran for their function. As with other nuclear transport receptors, XPO5 binds cooperatively to its cargo and the GTP-bound form of the cofactor Ran in the nucleus, and releases the cargo following the hydrolysis of GTP in the cytoplasm. Ran is a GTPase that binds in the GTP-bound form to karyopherins (XPO5 in this case) and forms a heterotrimer with pre-miRNAs [16, 17]. The XPO5/Ran-GTP heterodimer binds small RNAs bearing a terminal ≥ 16 nt and a short 3' overhang, i.e., precisely the structure of the pre-miRNA [42]. Knockdown experiments of XPO5 showed little effect on the level of expression of the pri-miRNAs, but did significantly reduce the level of expression of the mature miRNA [17]. Loss and mutation of XPO5 also lead to the nuclear retention of pre-miRNAs [43]. These results identify the major function of XPO5, as a pre-miRNA nuclear exporter, and define it as a cofactor for miRNA biogenesis and function [16, 17].

Once liberated in the cytoplasm, pre-miRNAs are subsequently processed into ~ 22 nt miRNA duplexes by the cytoplasmic RNase III Dicer together with its catalytic partner *Trans*-activator RNA (tar)-binding protein (TRBP) [44–48]. Dicer has a single processing center with intramolecular dimerization of the two RNase III domains. Each RNase domain cuts independently one RNA strand of the duplex and generates products with 2-nt 3' overhangs [49]. Mammalian cells have a single Dicer protein, with a molecular weight of ~ 200 kDa. Dicer contains an ATPase/RNA helicase domain, a DUF domain (a divergent dsRBD), a PAZ domain (a nucleic

acid-binding domain, with a strong preference for single-stranded nucleic acids or RNA duplexes with single-stranded 3' overhangs), two RNase III domains, and a dsRBD [44]. The PAZ domain of Dicer, also found in Argonaute proteins, is thought to bind to the 2 nt 3' overhang present at the base of the pre-miRNA hairpin, and the dsRBD of Dicer binds the stem and defines the distance of the cleavage from the base [50]. The two strands of the duplex are separated by Dicer and the strand whose 5' end forms the more unstable duplex with its partner seems to preferentially survive as the mature miRNA [27, 51–53]. Dicer knockout (*Dcr^{-/-}*) mice and cells are not viable, indicating a key role for this protein during developmental and normal cell function [44].

Dicer associates with several other proteins including TRBP and Argonaute family. TRBP protein is necessary for RNAi function; nonetheless TRBP is not required for the cleavage reaction itself; instead, it has various roles in maintaining Dicer stability and “dicing” action on the cytoplasm [54–57]. The TRBP C-terminal–Dicer interaction and its function as part of the RNA-induced silencing complex (RISC) have been identified as an important component of the RNAi pathway [58]. TRBP binds Dicer in the amino-terminal DExD/H-box helicase domain and activates Dicer through a conformational rearrangement [59]. Following Dicer/TRBP cleavage, the resulting ~22-nt RNA duplex is loaded onto Argonaute proteins so as to generate the effector complex, RISC. RISC is the cytoplasmic effector machine of the miRNA pathway and contains a single-stranded miRNA guiding it to its target mRNAs.

Cytoplasmic miRNA processing and RISC assembly are mediated by the RISC loading complex (RLC). RLC is a multi-protein complex composed of Dicer, TRBP, and the core component Argonaute-2 (AGO2), which also mediates RISC effects on mRNA targets [60, 61]. The slicer activity of AGO2 cleaves the 3' arm of the hairpin in the middle to generate a nicked hairpin, producing the AGO2-cleaved pre-miRNA [62].

Recently a Dicer-independent AGO2-dependent mechanism has been described for miR-451 [63, 64]. The precursor miR-451 (pre-miR-451) has an unusual secondary structure with a predicted stem of only 17 nt in length that seems to be too short to serve as substrate for Dicer, which requires >19 nt stem in addition to a 2 nt 3' overhang for efficient hairpin cleavage [65, 66]. Thus it has given rise to a conserved miRNA biogenesis pathway that requires AGO2 catalysis [63, 64]. The conserved miR-451 matures by direct cleavage of its corresponding pre-miRNA via the “slicer” activity of AGO2 [63, 64].

2.2.1.3 MicroRNAs and Posttranscriptional Regulation of Gene Expression

The small size of miRNAs provides a limited amount of sequence information for specificity. Partial base-pairing between a miRNA and its target mRNA is sufficient for repression and/or degradation of the target; thus a wide net of mRNAs can be regulated by the same miRNA. MiRNAs select mRNA targets for downregulation

through the association with a large, multi-protein complex, the RISC. This selection requires the presence of sequences within the target mRNA which are imperfectly complementary to the miRNA sequence. MiRNA-binding sites commonly occur within the 3'-untranslated region (3'-UTR) of the mRNA, but functional miRNA-binding sites can also occur with the 5'UTR or coding region [67, 68].

The 3'-UTRs of messenger RNAs serve as docking platforms for miRNAs and RNA-binding proteins, which control mRNA stability, localization, and translation [69, 70]. The most common feature is perfect base-pairing between nucleotides 2 and 7 at the 5' end of the miRNA, which is called the "seed" sequence, and the target site [69]. There is still a great deal of uncertainty regarding the exact composition of the miRNA complex as well as the mechanisms used to control target gene expression. What is known is that miRISC inhibits the expression of mRNAs basically in one of the two ways depending on the degree of complementarity between miRNA and the target. There are mismatches and bulges in most miRNA target sites; therefore the degree of complementarity between the miRNA and the target is thought to be a major determinant in distinguishing the two mechanisms of post-transcriptional silencing: translation inhibition or mRNA degradation. Several other mechanisms have been however documented, including translational inhibition at the level of initiation and elongation, rapid degradation of the nascent peptide, and mRNA degradation (Fig. 2.3). The core component of the mRNP complex is the Argonaute protein and in mammals one of the four Argonaute proteins (AGO1-4) is recruited into the complex. AGO2 is the only Argonaute with "slicer" activity and is responsible for the cleavage of the mRNA target midway into the complementary region. Other proteins are also essential for this silencing complex to work, such as the RNA-binding protein fragile-X-mental-retardation protein (FMRP), the p-body marker GW182, and the decapping activator RCK/p54, which likely dictate how the silencing of the mRNA target will occur [71–76].

2.3 Regulation of MicroRNA Biogenesis

Most miRNAs are under the control of developmental and tissue-specific signaling [77]. Stringent control of miRNA levels is crucial to maintain normal cellular functions, and deregulation of miRNA expression is often associated with human diseases, such as cancer [78]. Therefore it is highly important to have the maturation pathway of miRNAs subject to intense regulation at various levels, from stability, processing, sequence identity, and binding to target mRNAs. Likewise the miRNA biogenesis pathway is often subjected to feedback regulation. These autoregulatory feedback loops are a common mechanism particularly important in cell fate determination and development. MiRNAs are perfectly suited to participate in these networks owing to their potential to directly repress mRNAs that encode factors involved in the biogenesis or function of the same miRNAs. The control of the miRNA biosynthesis pathway is emerging as an important mechanism in defining the spatiotemporal pattern of miRNA expression in cancer cells. Interestingly transcriptional regulation is probably the main control mechanism.

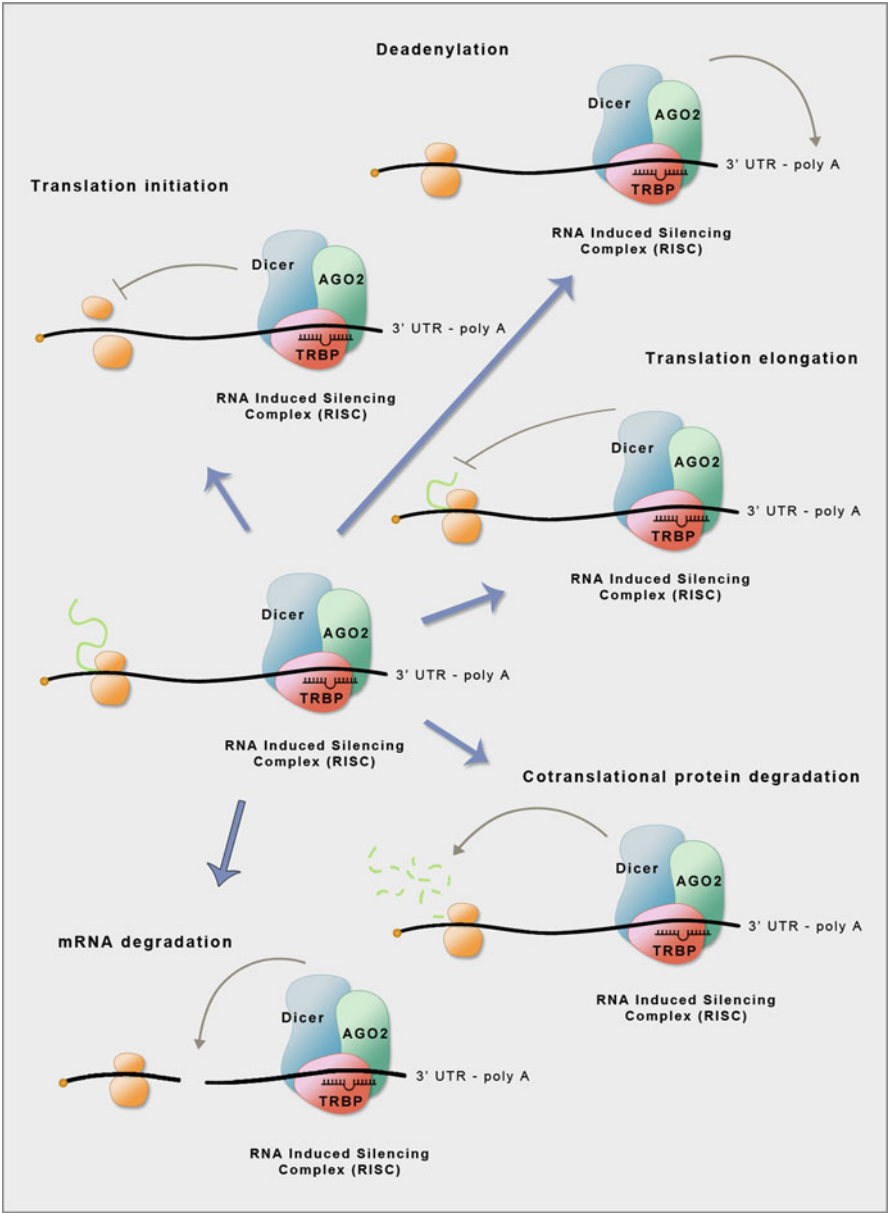


Fig. 2.3 Mechanisms of action of miRNAs. The silencing mechanisms modulated by miRNAs remain under debate. The ones clarified until now involve translational repression, degradation, and/or deadenylation of target mRNAs, as well as cotranslational protein degradation

2.3.1 Regulation of MicroRNA Transcription

Transcription is a major point of regulation in miRNA biogenesis. Many characteristics of miRNA gene promoters, such as the existence of CpG islands, TATA box, TFIIB recognition, initiator elements, and histone modifications, are similar to the promoters of protein-coding genes; therefore the same mechanisms of gene expression control are applicable to miRNAs [18, 19]. MiRNA genes can be transcribed by RNA polymerase II or III; as both RNA polymerases are regulated differently and recognize specific promoter and terminator elements, they facilitate a wide range of regulatory options. In addition, many miRNAs are encoded in the genome as clusters and can be transcribed as long polycistronic primary transcripts; however it has been shown that each miRNA located in the same genomic cluster can be transcribed and regulated independently [79, 80].

MiRNA genes also contain DNA-binding factors that can be regulated by the action of transcription factors such as p53 and c-myc, as well as MEF2, PU.1, and REST [81–84]. The tumor-suppressor p53 activates the miR-34 family of miRNAs, whereas the oncogenic protein c-myc transactivates or represses a number of miRNAs that are involved in the cell cycle and apoptosis [11, 85]. It is well demonstrated that c-myc binds to E-boxes and activates transcription of the miR-17-92 cluster [84]. Consistent with the c-myc activation in tumors, miR-17-92 is often highly expressed in tumors [86]. Moreover, c-myc is shown to increase transcription of Lin-28B that mediates the posttranscriptional repression of let-7 miRNA family members [87]. Furthermore, transcription activation can occur in response to growth factor stimulation, including platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), and bone-derived neurotrophic factor [19, 84, 88–90].

Epigenetic control also contributes to miRNA gene regulation through the methylation of CpG islands at the promoter of miRNA genes that silences their transcription [91–93]. MiRNA promoters are also regulated by histone modifications during development and disease [94].

2.3.2 Regulation of the Cropping, Dicing, and Slicing Events

Drosha processing (cropping) constitutes an important point of regulation. The total levels of Drosha and DGCR8 in the cell are tightly controlled and play a role in the regulation of pri-miRNA processing. DGCR8 protein has a stabilizing effect on Drosha through the interaction with its middle domain, whereas Drosha controls DGCR8 levels by cleaving hairpins present in the DGCR8 mRNA, thereby inducing its degradation [95, 96]. Therefore a tight equilibrium needs to exist between the amounts of Drosha and DGCR8 to maintain the processing of pri-miRNAs flowing. In addition SMAD proteins, activated by BMP/TGF β , interact with Drosha and DDX5/p68 stimulating Drosha processing [97]. Likewise it was recently described that the tumor-suppressor breast cancer 1 (BRCA1) accelerates the processing of pri-miRNAs by direct interaction with Drosha and DDX5 [98]. In addition the

authors described that BRCA1 recognizes the pri-miRNA secondary structure and directly binds the pri-miRNA before Drosha interaction [98].

In the cytoplasm accumulation of Dicer is dependent on its partner TRBP, and a decrease in TRBP leads to Dicer destabilization and pre-miRNA processing impairment [54, 55, 99]. Truncating mutations of TRBP that cause lower TRBP protein expression are associated with both a defect in the processing of miRNAs and destabilization of the Dicer protein [54]. TRBP itself is stabilized by phosphorylation mediated by mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) [99]. Notably, let-7 and miR-107 can target Dicer mRNA forming a feedback loop with the potential to influence miRNA biogenesis at the Dicer dicing step [68, 100].

Moreover, there are a significant number of RNA-binding proteins that influence miRNA processing at the Drosha and Dicer levels through specific interaction with a subset of pri-miRNAs or pre-miRNAs. The RNA-binding protein LIN28 is the best-studied negative regulator of miRNA biogenesis, which can act at different levels. LIN-28 binding to the terminal loop of pri-let-7 interferes with cleavage by Drosha; likewise binding of LIN-28 to the pre-let-7 can also block its processing by Dicer through polyuridylation of pre-let-7 performed by the enzyme TUT4 [101–105]. In addition, Kedde and colleagues demonstrated that the expression of dead end 1 protein (Dnd1), an evolutionary conserved RNA-binding protein, counteracts the function of several miRNAs in human cells and in primordial germ cells of zebrafish by binding mRNAs and prohibiting miRNAs from associating with their target sites. These effects of Dnd1 are mediated through uridine-rich regions present in the miRNA-targeted mRNAs [106]. Likewise, the RNA-binding protein RBM38 has been described to control miRNA access to target mRNAs [107]. Target selectivity is determined by the interaction of RBM38 with uridine-rich regions near miRNA target sequences [107].

RNA editing is another possible way of regulating miRNA biogenesis through the action of adenosine deaminases acting on RNA (ADARs), enzymes that modify adenosine (A) into inosine (I). Adenosine-to-inosine editing of pri-miRNAs can lead to changes in miRNA stability or alter their target selection [108]. Editing can also change the target specificity of the miRNA if it occurs in miRNA sequences [108].

2.4 Biological Roles of MicroRNAs

The importance of faithful miRNA expression has been involved in numerous cellular events during animal development where it is pivotal for the timing and regulation of many key processes such as cell fate determination, proliferation, and cell death [109]. In addition to these vital processes, miRNAs are implicated in other multiple biological functions, such as immune response, insulin secretion, neurotransmitter synthesis, circadian rhythm, and viral replication [110–113]. This list will undoubtedly expand as experimental data accumulates.

Representative examples of the wide scope of miRNA regulation are found in the miRNA let-7, a miRNA critical for developmental timing [5]; a developmentally regulated miRNA, bantam, that controls cell proliferation via regulation of apoptosis [114]; miRNAs that control ES cell differentiation by stabilizing the self-renewing versus differentiated cell fates [115]; and stem cell division [116]. Specifically, let-7 miRNA is essential for normal development and has a temporally regulated expression pattern. The misexpression of let-7 has been linked to several types of human cancer. Other examples are miR-196, which is involved in hind limb development [117], and the brain-specific miR-134, which is necessary for synaptic development and plasticity [118]. Skin differentiation is promoted by miR-203, which represses p63 in stratified epithelial tissues, while precise levels of miR-1 are critical in cardiogenesis [119]. Normal immune function is dependent on miR-155 and B-cell differentiation is controlled by miR-150-mediated repression of the transcription factor c-Myb [120, 121]. In addition, the pancreatic islet cell-specific miR-375 regulates insulin secretion by inhibiting myotrophin, a component of the exocytosis pathway [110].

Recent studies implicated miRNAs with several different diseases besides cancer. MiRNAs are not only required for the development of early embryonic stem cell survival and differentiation, but also plays an important role in maintaining the survival of mature neurons and their function. In neurological diseases, the loss of miR-20a/b-1 cluster has been implicated in Alzheimer's disease and the loss of miR-133b may contribute to the decrease in dopaminergic neurons seen in Parkinson's disease [122, 123]. In heart disease the expression of miR-21 in cardiac fibroblasts contributes to interstitial fibrosis and cardiac hypertrophy, while miR-1 and miR-133 in cardiomyocytes protect against hypertrophy [124]. It was also shown that autosomal dominant progressive hearing loss is a consequence of point mutations in the seed region of miR-96, a miRNA expressed in hair cells of the inner ear [125]. Therefore miRNAs have been found deregulated in a wide range of human diseases but specifically have been described to play an important role in human cancer. However, it remains uncertain whether altered miRNA expression is a cause or a consequence of pathological processes.

2.5 MicroRNA Biogenesis and Cancer

2.5.1 *Oncogenic or Tumor-Suppressor MicroRNAs*

In line with their broad effects, miRNAs have been proposed to function as oncogenes or tumor-suppressor genes given their inhibition of a variety of tumor-suppressive and oncogenic mRNAs, respectively [126, 127]. Overexpressed miRNAs in cancer, such as miR-17-92, may function as oncogenes and promote cancer development by negatively regulating tumor-suppressor genes and/or genes that control cell differentiation or apoptosis. Non-expressed or low-expressed miRNAs in cancer, such as let-7, function as tumor-suppressor genes and may inhibit

cancers by regulating oncogenes. In particular, three distinct mechanisms have been posited. First, oncogenic miRNAs can undergo a gain of function in tumors. This has been more clearly demonstrated for the miR-17~92 cluster, whose amplification in B-cell lymphomas promotes their development, potentially through its control of B-cell differentiation [11, 128]. Furthermore, tumor-suppressive miRNAs could undergo loss of function in tumors. This has been shown for several miRNAs, including the let-7 family, whose expression can limit tumorigenesis through inhibition of oncogenes like the *RAS* family and *HMGA2* [129, 130]. In particular, let-7 family members are in sites frequently deleted in human tumors, and their processing is inhibited by the oncogenic Lin-28 proteins [103, 131, 132]. Finally, oncogenes can acquire mutations to remove miRNA-binding sites in tumors. This has been described for *HMGA2*, whose translocation promotes lipoma development by releasing the transcript from let-7-mediated tumor suppression [133]. Furthermore, Voorhoeve and colleagues have identified miR-372 and miR-373 as oncogenes in human testicular germ cell tumors by numbing the p53 pathway [134]. These miRNAs neutralize p53-mediated CDK inhibition, possibly through direct inhibition of the expression of the tumor-suppressor LATS2 [134].

2.5.2 Impairment of MicroRNA Biogenesis in Cancer

Widespread downregulation of miRNAs is a common feature of human cancers when compared with normal tissues [135]. Whether this tendency is a reflection of a pattern associated with specific cells of origin, is a consequence of the malignant state, or actively contributes to cancer development is still unclear. Two main mechanisms have been proposed as the cause of the global downregulation of miRNAs in cancer. One is related with transcriptional repression by oncogenic transcription factors like MYC oncoprotein [85]. The other mechanism involves impaired miRNA biogenesis and is based on the observation that cancer cells often display reduced levels of proteins involved in the miRNA biogenesis pathway [136]. In addition, global repression of miRNA biogenesis by suppression of the key components of miRNA processing machinery, such as Drosha, DGCR8, Dicer, TRBP, and XPO5, promotes cellular transformation and tumorigenesis [43, 54, 85, 137]. While the mechanism(s) remains to be fully elucidated, it suggests that miRNAs might have an intrinsic function in tumor suppression and its downregulation eventually accelerates oncogenesis.

After transcription, global levels of miRNAs can be reduced by impaired miRNA biogenesis. Inactivating mutations and reduced expression have been described for almost all the members of the miRNA processing machinery. Furthermore the existence of frameshift mutations in the TARBP2 and XPO5 genes in cancer cell lines and primary tumors has been recently reported [43, 54, 138]. These genetic alterations in miRNA biogenesis genes are associated with nonfunctional truncated forms of both proteins. The first mutation to be described was in the TARBP2 gene that codes for the TRBP protein, the catalytic partner of Dicer. Frameshift mutations in

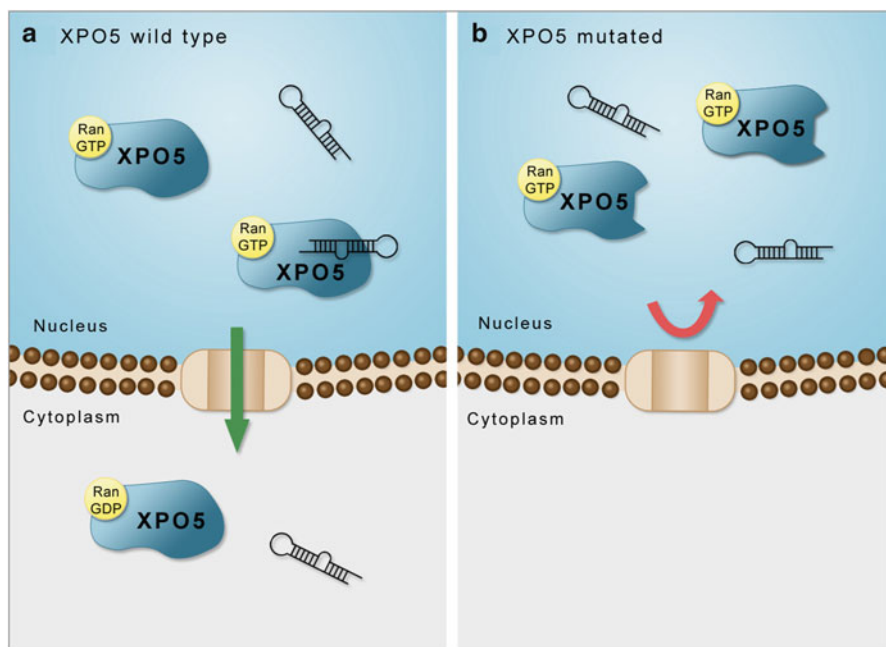


Fig. 2.4 Impairment of miRNA biogenesis in cancer at the exportin-5 step. **(a)** In a normal setting the formation of a heterotetrameric complex between XPO5/Ran/GTP/pre-miRNA results in export of the pre-miRNA from the nucleus to the cytoplasm to be further processed. **(b)** In cancer, heterozygous frameshift mutations in the XPO5 gene lead to a truncated form of the XPO5 protein that does not recognize/bind the pre-miRNA molecule, therefore preventing the formation of a functional XPO5/Ran/GTP/pre-miRNA complex and its translocation from the nucleus to the cytoplasm (*right panel*)

TARBP2 result in monoallelic expression of wild-type TRBP. Thus, due to TRBP downregulation Dicer protein is destabilized slowing down the dicing process of pre-miRNAs culminating in a global downregulation of miRNAs due to impaired processing [54]. Likewise, the mutations found in exon 32 of XPO5 alter and truncate the protein sequence and prevent XPO5 from associating with its pre-miRNA cargo and exiting the nucleus (Fig. 2.4). In XPO5 heterozygous mutant cells, less pre-miRNA was accessible to processing by the cytoplasmic machinery, resulting in decreased mature miRNA levels and enhanced tumorigenicity. Many pre-miRNAs are also targeted by ADARs at various stages of their processing, and the modification can also prevent export of pre-miRNAs. In addition germline truncating mutations in Dicer, that is essential for processing miRNAs, have been observed in families with the pleuropulmonary blastoma family tumor and dysplasia syndrome [139]. Recent work has suggested that other components of the miRNA biogenesis pathway, Dicer and TARBP2, are haploinsufficient tumor suppressors [54, 140]. Moreover, biallelic deletion was found to impair cell viability, hence preventing the phenomenon of loss-of-heterozygosity (LOH) [140]. This is also the case for XPO5,

where mimicking LOH by RNA interference against the XPO5 wild-type transcript rendered cells unviable [43]. In addition, the miRISC components AGO2, TNRC6A, and TNRC6C can also be mutated in cancer, although the functional consequences remain to be evaluated [138].

Two other groups have also reported the accumulation of let7 miRNA precursors at various stages during fruit fly and sea urchin development [46, 141]. Although this could represent a defect in RNA processing, it is also possible that the nuclear export of the let7 precursor, and hence access to cytoplasmic Dicer and TRBP, is developmentally regulated.

Hence, it is possible that global dys-regulation of miRNAs in cancer has its roots in impaired miRNA processing through genetic mutations of their components contributing to cancer progression.

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