

MicroRNAs: molecular features and role in cancer

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1. ABSTRACT

microRNAs (miRNAs) are small noncoding endogenously produced RNAs that play key roles in controlling the expression of many cellular proteins. Once they are recruited and incorporated into a ribonucleoprotein complex miRISC, they can target specific mRNAs in a miRNA sequence-dependent process

and interfere in the translation into proteins of the targeted mRNAs via several mechanisms. Consequently, miRNAs can regulate many cellular pathways and processes. Dysregulation of their physiological roles may largely contribute to disease. In particular, in cancer, miRNAs can be involved in the deregulation of the expression of important genes that play key roles in tumorigenesis, tumor development, and angiogenesis and have oncogenic or

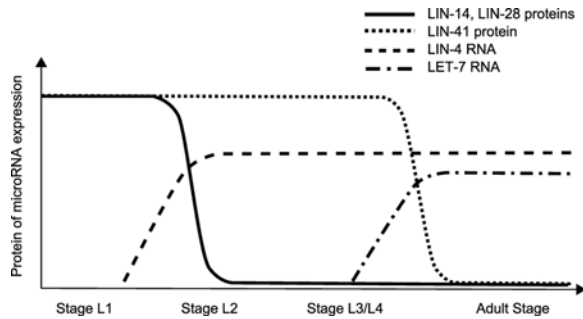


Figure 1. Time-dependent expression of lin-4 and let-7 microRNAs during *C. elegans* larval development and their impact on the expression of key proteins involved in larvae stage transitions.

tumor suppressor roles. This review focuses on the biogenesis and maturation of miRNAs, their mechanisms of gene regulation, and the way their expression is deregulated in cancer. The involvement of miRNAs in several oncogenic pathways such as angiogenesis and apoptosis, and in the inter-cellular dialog mediated by miRNA-loaded exosomes as well as the development of new therapeutical strategies based on miRNAs will be discussed.

2. INTRODUCTION

miRNAs were described in *Caenorhabditis elegans* at the beginning of the 1990s (1) as small noncoding endogenously produced RNAs that inhibit mRNA translation. Nevertheless, the existence in eukaryotic cells of RNA molecules called tcRNAs (translational control RNAs), that were able to inhibit the translation of mRNAs, had been previously suggested by Heywood et al. (2). The first miRNA described in *C. elegans*, lin-4, is a small RNA containing complementary sequences for repeated elements of the 3'UTR region of the lin-14 and lin-28 mRNAs. Lee et al. proposed a lin-4-dependent regulation of the translation of the lin-14 and lin-28 mRNAs due to a sense-antisense RNA interaction. This regulation model was validated by Wightman et al. who used reporter genes and demonstrated that the 3' untranslated region (UTR) of lin-14 mRNA was essential for the regulation of its translation by lin-4 (3). A second small RNA, let-7, which, similar to lin-4, comes from the family of heterochronic switch genes, and controls the larval development of *C. elegans* was then discovered by Reinhart et al. (4). let-7 plays a role in the transition of the larval L3/L4 stage to the adult stage, repressing the expression of LIN-41 by interacting with the 3'UTR region of the mRNA.

lin-4 and let-7 were first called small temporal RNAs (stRNAs) (5) due to their role in the control of development and were renamed miRNAs in 2001 (6) (Figure 1). Afterwards, several miRNAs were described in other organisms such as *Drosophila melanogaster*, mice, and plants as well as in human cell lines (7-13).

3. miRNA: NOMENCLATURE, BIOGENESIS AND FUNCTIONS

3.1. miRNA nomenclature

miRNAs are identified either by cloning and sequencing studies or informatics approaches using prediction programs based on conservation of miRNA within species and on the detection of the stem-loop structures typical of miRNA precursors. All miRNAs named in scientific publications and generally found using cloning and sequencing techniques are listed in the database called miRBase (<http://microrna.sanger.ac.uk/> (14)). miRBase version 18 (November 2011) now classifies 1527 miRNAs for humans.

miRNA-encoding genes are first transcribed in stem-loop structured primary transcripts called pri-miRNAs, which are then excised by RNase III Drosha and its cofactor DGCR8, freeing a precursor called pre-miRNA. These precursor molecules are then exported from the nucleus to the cytoplasm with exportin-5. They are then matured by the RNase III Dicer to free a duplex miRNA-miRNA*. One strand, called mature, is then incorporated into the RISC complex, a complex containing essentially Argonaute-type proteins. (Figure 2).

While genes or pre-miRNAs are noted “mir-X”, mature miRNAs are noted “miR-X” where X is a numeric value. A three-letter code is added as a prefix to refer to the organism they originate from. One letter stands for the organism genus and the two others for the species, e.g., hsa for *Homo sapiens* or mmu for *Mus musculus*.

When mature miRNAs differ by a few bases, a letter is added as a suffix, reflecting the difference: e.g., miR-181a and miR-181b:

- miR-181a: acaauucaacgcugucggugagu
- miR-181b: acaauucauugcugucggugggu

When both strands of a pre-miRNA are processed as mature miRNAs, an indication is given to specify which arm of the hairpin structure of the pre-miRNA generates one or the other of the two miRNAs. Several nomenclatures can be used:

- miR-X and miR-X*: miR-X* is the less abundant of the two mature miRNAs in the cells.
- miR-X-5p and miR-X-3p: 5p and 3p stand for the 5' and 3' arms of the stem-loop structure of the pre-miRNA, respectively. This nomenclature is mainly used when the respective abundance of the two miRNAs is still unknown (15).
- miR-X-s (5' arm) and miR-X-as (3' arm).

Moreover, a mature miRNA can result from the transcription and maturation of transcripts from separate genomic loci. A numeral suffix is added to the names of all the multiloci miRNAs, e.g., miR-181a-1 and miR-181a-2 (miR-181a-1 originates from a locus on chromosome 1 and miR-181a-2 from a locus on chromosome 9). Nevertheless,

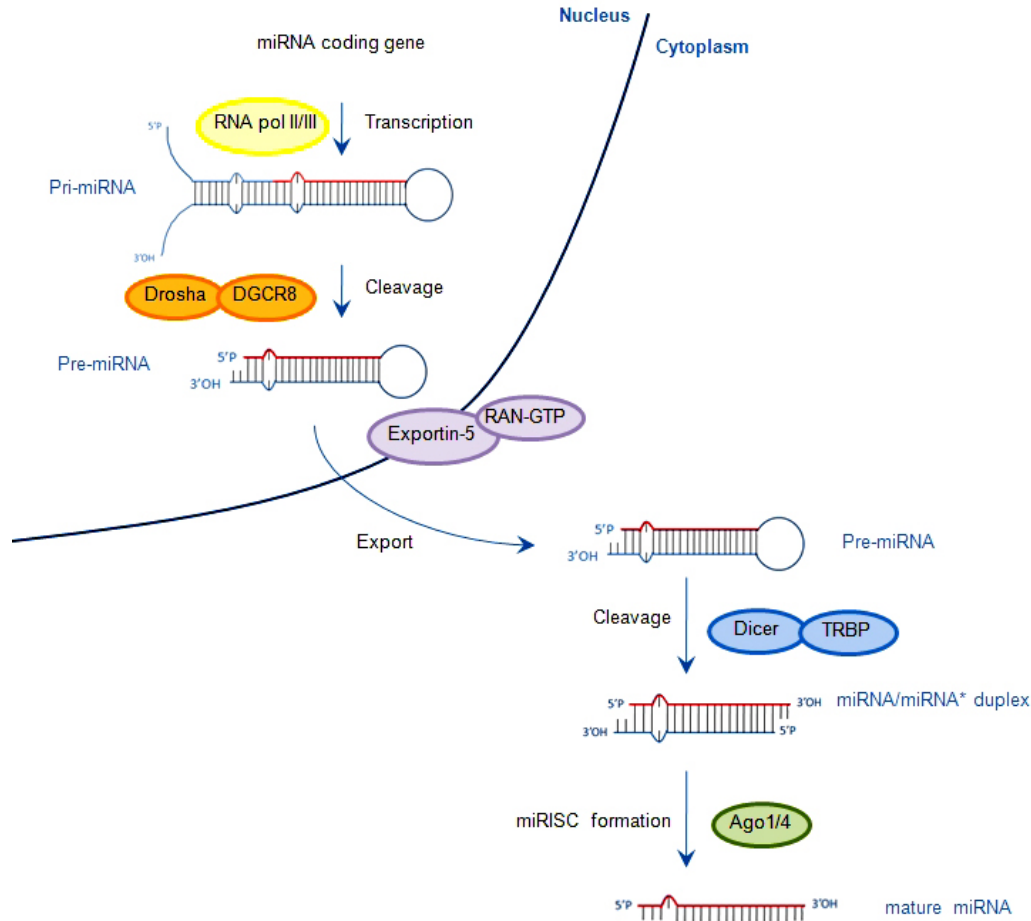


Figure 2. The biogenesis mechanism of microRNAs. The transcription of a miRNA coding gene by a polymerase (yellow symbol) or the splicing of an intron generates a primary transcript called pri-miRNA. The cleavage of this transcript by the RNase Drosha associated with DGCR8 (orange) generates an RNA precursor called pre-miRNA. This precursor is then exported to the cytoplasm *via* the exportin-5 (purple) where it will be excised by the Drosha enzyme associated with the TRBP protein (blue). Only one of the two duplex strands of the miRNA, called the mature strand, is incorporated into the RISC complex, mainly composed of Argonaute proteins (green).

some miRNAs, such as hsa-let-7 and cel-lin-4, do not follow the above-described nomenclature.

3.2. Interfering RNAs: similarities and differences

RNA interference (RNAi) is mainly responsible for the post-transcriptional regulation of gene expression (mRNA cleavage or decay, translation repression or activation) but is also involved in transcriptional regulation as described by various studies (16-19).

Mainly two classes of small RNAs interfere in the RNA interference process: miRNAs and siRNAs (short interfering RNA). These RNAs share similarities such as their short size (about 22 nucleotides), their maturation by the ribonuclease III Dicer, and their implication in the RISC complex for post-transcriptional repression.

Nevertheless, there are a number of important differences, such as the following:

- miRNAs are exclusively endogenously produced. They come from intrinsic genes of the organism they interfere in, whereas siRNAs may originate from viruses, transposable elements, perfectly paired endogenous double-stranded RNA resulting from an antisense transcription of various loci (20), or transfected exogenous and double-stranded RNAs.
- miRNAs play a role in the regulation of genes that do not generate them, whereas siRNAs mainly regulate the genes that produce them. This point is critical in order to secure host cell defense against parasite RNAs such as viruses or transposons.
- In mammals, miRNAs have a varying degree of complementarity with their target, whereas siRNA always have a perfect complementarity with their targets. When the complementarity is perfect, the target mRNA is degraded in the presence of the Ago2 protein, which is the sole protein of the four Argonaute family proteins responsible for endonucleolytic cleavage. In plants, the difference between

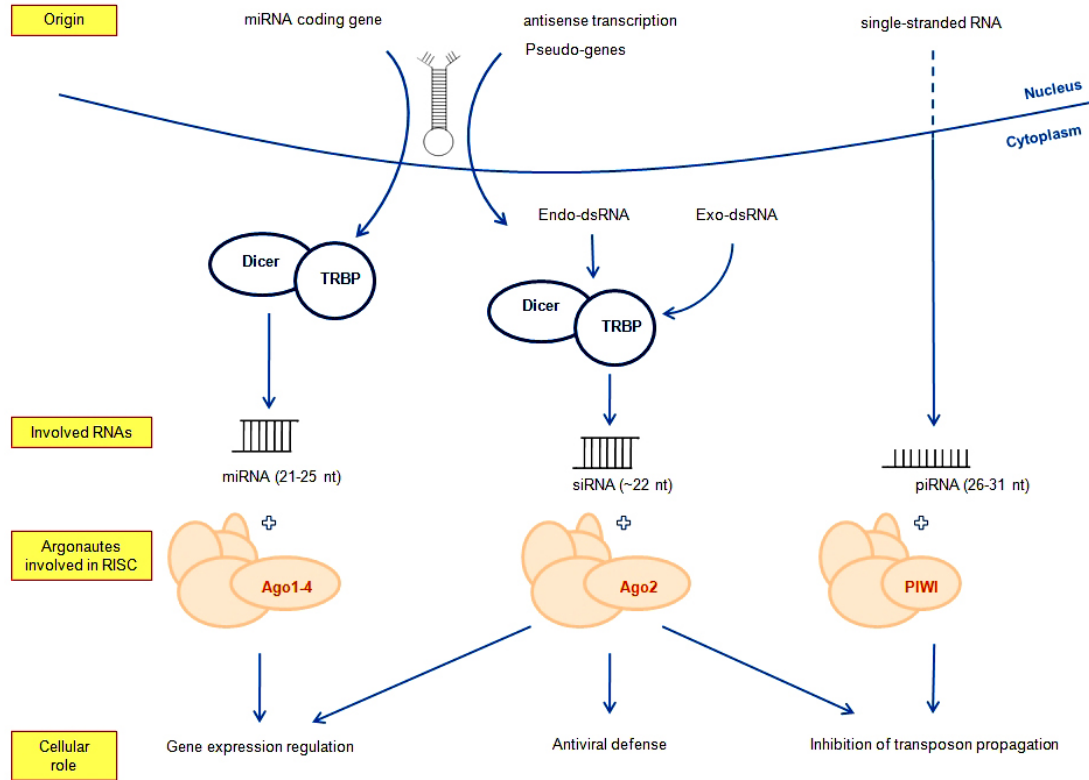


Figure 3. Comparison of miRNAs, siRNAs, and piRNAs. miRNAs are processed according to the pathway described in Figure 2. siRNAs come from exogenous double-stranded RNA (Exo-dsRNA; viral infection) or endogenous double-stranded RNA (Endo-dsRNA; reverse transcription or transposons). Like miRNAs, siRNAs are matured by Dicer associated with TRBP and then incorporated into the RISC complex mainly composed of Ago2. siRNAs play a role in cell defense after a viral infection, in the inhibition of transposon propagation, or in the simple regulation of gene expression. piRNAs are different from miRNAs and siRNAs mainly because of their origin and their maturation; they are derived from single-stranded RNAs and are not processed by Dicer. They are also a few nucleotides longer than miRNAs and siRNAs, are mainly present in germinal cell lines, and contribute to maintaining the genome integrity targeting transposable elements.

miRNAs and siRNAs is very small because the pairing between miRNA and mRNA is often perfect (21).

In addition to miRNAs and siRNAs, there are other small RNAs that contribute to maintaining the genome integrity: the piRNAs (piwi-interacting RNAs). They are longer (26–31 nucleotides), abundant in germinal cells but can also be found in somatic tissues (22–24) (Figure 3). These RNAs, formerly called repeat-associated siRNA (rasiRNA), were discovered in *D. melanogaster* (25). In 2006, several studies showed the presence of piRNAs in mammals (25–28). Contrary to siRNA and miRNA, it seems that they come from long single-stranded RNA precursors and their biogenesis does not include the RNase III Dicer. Once matured, piRNAs associate themselves with PIWI proteins, a subfamily of Argonaute proteins, principally to target transposons and preserve genome integrity (22).

3.3. miRNA biogenesis

3.3.1. Localization and structure of miRNA coding genes

These genes are widespread on all chromosomes except for chromosome Y and approximately 50% of the

described miRNAs are located in clusters. When these clustered miRNAs are under the control of a single promoter, they are dubbed polycistronic miRNAs.

miRNA coding genes can be independent gene units and miRNAs are then called intergenic; they can also be gene units coding for pre-mRNA that either code for proteins or do not code for them. In this case, miRNAs can be intronic or exonic (Figure 4) (29–31).

3.3.2. Transcription of primary miRNA transcript

Most of the miRNA coding genes are transcribed by the RNA polymerase II, producing long primary transcripts (pri-miRNAs). These pri-miRNAs have a typical structure composed of a stem (about 33 nucleotides), a loop connecting the two strands of the stem, and single-stranded flanking sequences (Figure 5). Like mRNAs, these sequences contain a 7-methyl-guanosine (m7G) cap at their 5' end and a 3' polyA tail (32, 33). With the transcription performed by the RNA polymerase II, the regulation of this mechanism could be similar to the regulation of the transcription of protein-coding genes. The localization of promoter regions of miRNA coding genes

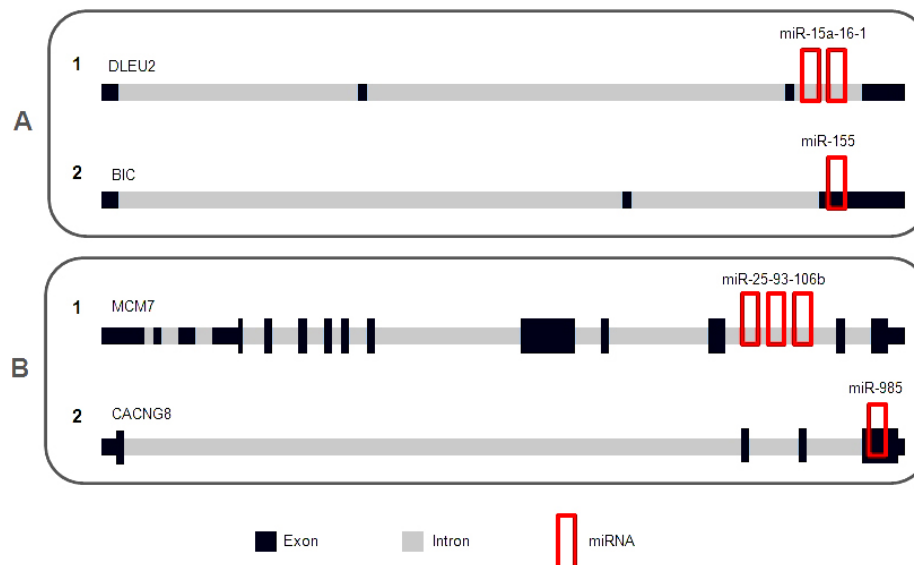


Figure 4. Localization of miRNA coding genes located in gene units coding for pre-miRNAs (31). miRNAs can be located in transcription units not coding for proteins (A) or coding for proteins (B). miRNAs can also be located in either the intron (1) or exon (2).

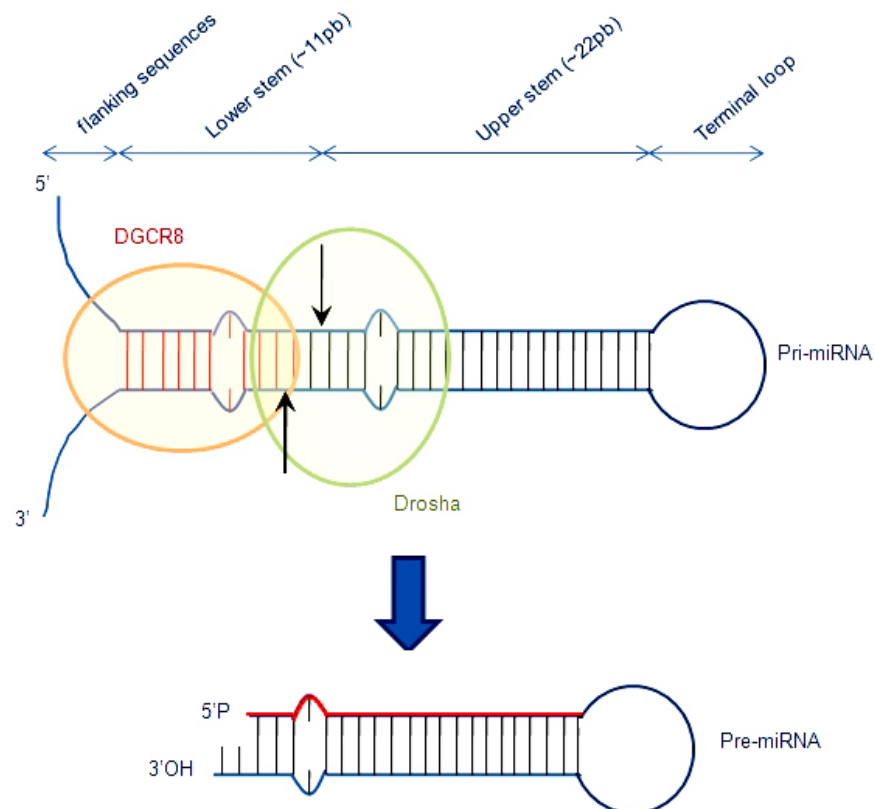


Figure 5. Maturation of pri-miRNA into pre-miRNA by the Drosha/DGCR8 complex (43). DGCR8 binds to double-stranded RNA, recognizes the pri-miRNA flanking sequences, and allows the stem-loop cleavage by the ribonuclease Drosha, approximately 11 bp from the junction between the flanking sequences and the stem-loop. This asymmetric cleavage leaves two unpaired nucleotides at the 3'OH end.

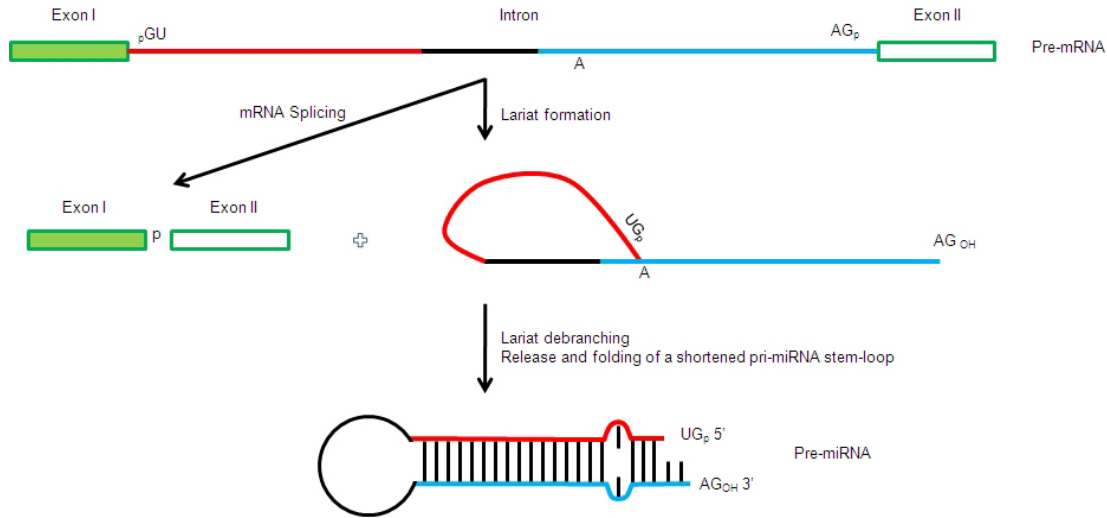


Figure 6. mirtron pathway (44,45). In the nucleus, short introns with hairpin potential, termed mirtrons, can be spliced and debranched into pre-miRNA hairpins. Hairpin potential is due to the complementarity of the sequences of the 5' and 3' domains of the introns (red and blue domains of the intron in the figure, respectively). The intron donor splicing site (GU) and the acceptor splicing site (AG) constitute the 5' end and the 3' end of the pre-mirtron, respectively.

was predicted *in silico* by genomic mapping of the transcription start sites (TSS) and by mapping of transcription factor binding motifs. This was reported in *Arabidopsis thaliana*, *C. elegans*, and *H. sapiens* (34-36). Furthermore, two studies highlighted the localization and characteristics of TSS sequences of miRNAs in human using high-throughput genomic analyses such as chromatin immunoprecipitation (37, 38).

3.3.3. Maturation of pri-miRNAs into miRNAs

3.3.3.1. Formation of the pre-miRNA in the nucleus

In human, primary transcripts are processed in the nucleus by a protein complex called microprocessor that is composed of the ribonuclease Drosha associated with its cofactor DGCR8 (DiGeorge syndrome Critical Region gene 8) (39). The Drosha complex also contains several other factors such as EWSR1, FUS, numerous heterogeneous nuclear ribonucleoproteins (hnRNPs), p68 (DDX5) and p72 (DDX17) DEAD-box helicases. The roles of these accessory components in miRNA maturation is largely unknown, some hnRNPs and p68/p72 may promote the fidelity and activity of the Drosha processing (40, 41). DGCR8, which interacts with double-stranded RNA, is required to allow the binding of the Drosha/DGCR8 complex to the pri-miRNA at the junction between the flanking sequences and the stem-loop. Once the complex is bound to the pri-miRNA, DGCR8 specifies the Drosha cleavage distance (~11 bp) away from the junction between the flanking sequences and the stem-loop. The Drosha enzyme is a class 2 ribonuclease III, *i.e.*, it contains two RNase III domains and one binding domain for double-stranded RNA. This structure allows an asymmetric cleavage of the double-stranded RNA and the release of a shortened pri-miRNA stem-loop (42). This shortened stem-loop is called pre-miRNA and contains two unpaired nucleotides at the 3' end and a phosphate group at the 5' end (43) (Figure 5).

Another pre-miRNA biogenesis pathway is the mirtron pathway, which is connected to the mRNA splicing mechanism. After splicing and lariat release, the intron structures itself in a stem-loop to generate a pre-miRNA. In this case, the mechanism of mirtron maturation bypasses cleavage by the pre-miRNA generating enzyme Drosha. The pre-miRNA 5' and 3' ends correspond to the donor splicing site (GU) and the acceptor splicing site (AG), respectively. This mirtron pathway, first described in the invertebrates *D. melanogaster* et *C. elegans* (44, 45), was then evidenced in mammals (such as miR-877 and miR-1224) (46). The main difference between the invertebrate and mammal mirtron pathways seems to rely on the fact that the mature miRNA is usually located on the 3' strand of the stem-loop in invertebrates while it is located on the 5' strand in mammals (Figure 6).

3.3.3.2. Pre-miRNAs export from the nucleus to the cytoplasm

The export of RNAs (messenger, ribosomal, and transfer RNAs) is made possible by the action of receptors of the karyopherin family, such as CRM1 and exportin-t (Exp-t). Nevertheless, nuclear-export of pre-miRNAs mostly relies on another receptor called exportin-5 (Exp-5) (47, 48). This receptor, discovered in 2002, belongs to the karyopherin beta family. Like all the other receptors of this family, it binds directly and specifically to the RAN-GTP protein (RAS-related nuclear protein with bound GTP) in the nucleus and then binds to nucleoporins to allow the pre-miRNA transfer from the nucleus to the cytoplasm (49). Exp-5 specifically interacts with double-stranded RNAs longer than 14 bp that contain a protruding 3' end (50), hence its binding specificity for pre-miRNAs (51). Once the pre-miRNA/Exp-5/RAN-GTP complex is assembled and is bound to nucleoporins, it goes through the nuclear pores. Then the RAN-GAP enzyme (RAN-GTPase activating protein), located on the cytoplasmic side

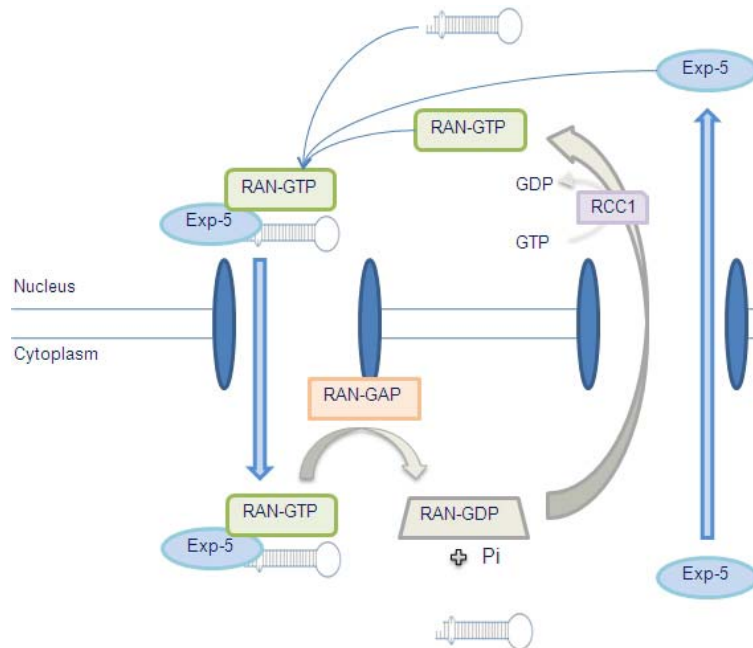


Figure 7. Mechanism of pre-miRNA export from the nucleus to the cytoplasm (48). The Exp-5/RAN-GTP/pre-miRNA complex binds to nucleoporins in the nucleus and goes through the nuclear pores. The release of pre-miRNAs into the cytoplasm is linked to the hydrolysis of RAN-bound GTP by the RAN-GTPase-activating protein. Exp-5 is then transported back into the nucleus and RAN-GTP is regenerated with RCC1 (Ran guanine nucleotide exchange factor).

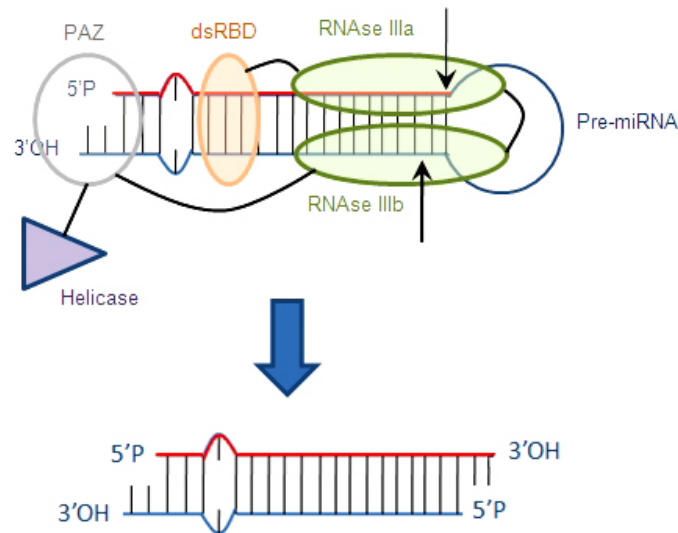


Figure 8. Dicer-dependent cleavage of pre-miRNA (59). The Dicer PAZ domain recognizes the single-stranded 2 nucleotide-long 3' overhang of the pre-miRNA. The dsRNA binding domain (dsRBD) allows the positioning of the RNase III domains and the cleavage of the pre-miRNA.

of the pores, activates the hydrolysis of the RAN-bound GTP into RAN-bound GDP (52), leading to the release of the pre-miRNA in the cytoplasm (Figure 7).

3.3.3.3. Mature miRNA production in the cytoplasm

In the cytoplasm, pre-miRNAs become the substrates of the ribonuclease III Dicer, which completes their processing. Previously described for siRNA processing (53), Dicer was found to be involved in the final cleavage of pre-

miRNAs into 21-nucleotide miRNAs in *C. elegans* (54, 55). Dicer is a class 3 type III ribonuclease composed of two RNase III domains, one double-stranded RNA (dsRNA) binding domain, one PAZ signature (Piwi/Argonaute/Zwille), and a helicase domain. The PAZ domain allows Dicer to recognize the single-stranded 3' overhangs of the pre-miRNA (56, 57). In addition, the dsRNA binding domain allows the recognition of the stem by Dicer (58, 59) and the cleavage by the RNase III domains of the dsRNA about 21 nt away from

the PAZ-bound end. This cleavage generates another single-stranded 3' overhang (Figure 8).

3.4. Gene expression regulation by miRNAs

3.4.1. miRISC (miRNA-induced silencing complex) formation

During the maturation step catalyzed by Dicer, two other proteins, TRBP (TAR (HIV-1) RNA binding protein or TARBP2) and Ago2 (Argonaute 2), were found to be involved in the formation of the RISC loading complex (RLC) with Dicer (60-63). Most of the studies carried out *in vitro* with recombinant proteins revealed that the association of only three proteins – Dicer, TRBP, and Ago2 – makes an equistoichiometric complex that is able to process the pre-miRNA and to select one of the two strands to form the miRISC. The dimeric TRBP (64) first dissociates into monomers to form the RLC. In *Drosophila*, the assembly of the Dicer/TRBP/Ago complex requires ATP (65), whereas in human, assembly of the complex can be either ATP-independent (61-63) or ATP-dependent (66).

The RNA duplex obtained after maturation contains 2-nt 3' overhang ends, resulting from the action of the two type III RNases, Droscha and Dicer. In some rare cases, the two duplex strands can accumulate in the cell (6, 67) with both strands acting as mature strands to guide the miRISCs to the mRNA targets (68). Nevertheless, most of the time only one of the two strands is selected according to asymmetric rules (69, 70). The unselected strand is called the passenger strand and noted miR-X* (see nomenclature Part 3.1). The selected strand is called the guide strand or mature strand and noted miR-X. This strand has the less thermodynamically stable 5' end, *i.e.*, with the weakest 4-bp-long duplex binding energy (70). Indeed, this thermodynamic instability can facilitate the unwinding of the duplex by helicases, such as Gemin3 (67) or RCK/p54 (71). Unwinding of the duplex may also be promoted by Dicer's helicase domain, since it was shown that it can be performed by the *in vitro* associated RLC (only containing Dicer/TRBP/Ago2) (63). ATP does not seem to be necessary for the dissociation of the two strands by a helicase. Indeed, the energy released after the cleavage of the two phosphodiester bonds by Dicer could provide energy to achieve this dissociation step (63). Another recent study suggests that Ago1 and Ago2 may participate in the separation of the duplex's two strands (72). For siRNA duplexes, after the dissociation of the two strands, the passenger strand is degraded by cleavage by Argonaute proteins (73, 74). As for the miRNAs duplexes, the fate of the passenger strand seems less clearly identified. According to Matrangola *et al.*, degradation by cleavage does not occur because of the imperfect complementarity between the two strands (75). According to Shin *et al.*, the passenger strand of a miRNA duplex may be degraded if strong complementarity is observed in the middle part of the duplex (76). Another pathway has also been proposed by Diederichs and Haber, who claim that the passenger strand is cleaved by Ago2 before Dicer's action (77).

RLC allows *in fine* the loading of the mature miRNA into the miRISC after the dissociation of the two

strands. Among the three proteins that take part in the RLC (Dicer, TRBP, and Ago), only the Ago component is required for RISC activity in the miRISC (Figure 9). Argonaute proteins are composed of two main regions: one containing the N-terminal and the PAZ domains, the other one containing the Mid and PIWI domains (78). Although the functions of these domains are poorly defined, it seems that the three domains – PAZ, Mid, and PIWI – play an important role in the RNA interference phenomenon as they are required for the interaction with the miRNA. Indeed, the PAZ domain, which is similar to the Dicer domain, binds to RNA duplexes containing two unpaired nucleotides on the 3' end and therefore recognizes the 3' end of mature miRNAs (56, 57, 79). The Mid domain recognizes the phosphate 5' end of mature miRNAs and also mRNA m7G capping (80, 81). Finally, the PIWI domain contains a tertiary structure specific of the RNase H family providing an endonucleolytic activity to RISC (82). In *Drosophila*, only Ago1 is specialized in the loading of miRNAs (Ago2 is specialized in loading siRNAs) (83). In human, there are four Argonaute proteins (Ago1–4). All four forms can load miRNAs, but only Ago2 has an endonucleolytic activity (84, 85).

3.4.2. Interaction between miRNAs and mRNA targets

3.4.2.1. Mechanisms of interaction

The main interaction between miRNAs and target messenger RNAs occurs at the level of the mRNA 3'UTR and generates duplexes. In plants, most miRNAs show a perfect complementarity with their mRNA targets and perfect duplex formation is associated with the degradation of the targets by endonucleolytic cleavage (86, 87). On the contrary, in metazoans, a perfect complementarity between miRNAs and their mRNA targets is not always the rule.

Several specific features of duplex formation have been described which were used to define algorithms to predict miRNA targets. In 2003, Lewis *et al.* reported that the hybridization between miRNA and its mRNA target has to be perfectly complementary between nucleotides 2 and 7 from the 5' end of the miRNA; this region was called the seed region (88). Any mismatch in this region and in particular any G:U type mismatch would more or less severely reduce the miRNA efficiency to interact with a given mRNA target, depending on the size of the seed region (89, 90). For example, with a 6-nt seed region, a G:U type mismatch leads to a complete loss of the miRNA activity. In addition, the presence of an A residue in position 1 and/or an A/U residue in position 9 from the 5' end of the miRNA (*i.e.*, on both sides of the seed region) may improve the interaction between miRNA and the targeted mRNA (91).

Another feature is the presence of a mismatched bulge in the middle region of the miRNA–mRNA duplex, which prohibits the endonucleolytic cleavage catalyzed by Ago2 (92). In addition, a sequence complementarity on the 3' end of the miRNA stabilizes the miRNA–mRNA duplex, especially when it occurs between nucleotides 13 and 16 from the 5' end of the miRNA (93).

Finally, other factors can improve the miRNA efficiency such as AU-repeat mRNA sequences close to

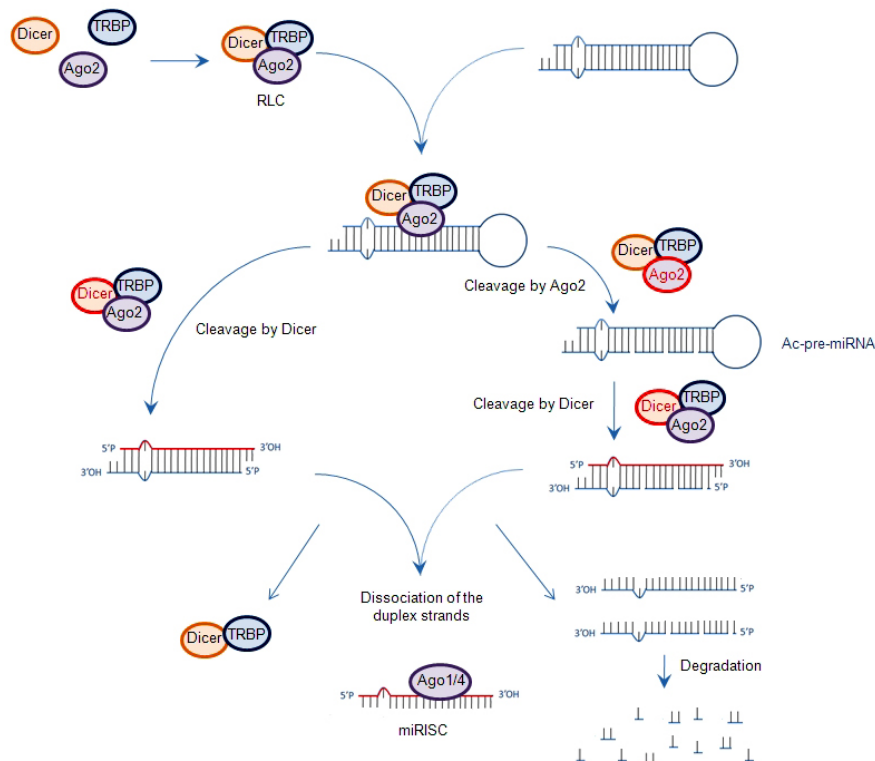


Figure 9. miRISC formation (77). The RLC is composed of Dicer, TRBP, and Ago2. Two pathways may lead to the formation of miRISC. One (left) includes dissociation of the two strands of the duplex after cleavage of the pre-miRNA by Dicer. The second (right) first relies on the cleavage of the complementary strand by Ago2, allowing the formation of a transitory miRNA called Ac-pre-miRNA (Ago2-clived pre-miRNA) before cleavage by Dicer. In this model, the passenger strand is then degraded.

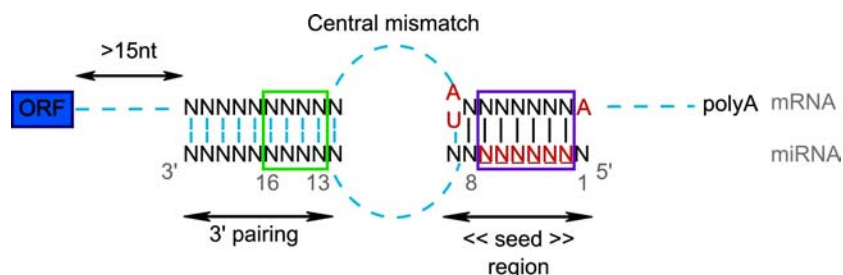


Figure 10. Schematic model of the interaction between miRNAs and target mRNAs in metazoans (94). The major structural features are the following. miRNAs target mRNAs in the 3'UTR at a distance from the stop codon greater than 15 nt. A perfect complementarity is found in the seed region between nucleotides 2 and 7 from the 5' end of the miRNA (pink rectangle). A central bulge prevents endonucleolytic cleavage mediated by Ago2 (a major difference with the miRNA–target mRNA interaction in plants). A few nucleotide matches in the miRNA 3' end (especially between nucleotides 13 and 16) is necessary for the best stabilization of the miRNA/mRNA target duplex (green rectangle). The presence in the mRNA sequence of an A residue in position 1 and/or an A or U residue in position 9 could increase miRNA efficiency.

the miRNA binding site, or the proximity of the miRNA site to the stop codon or to the polyA tail of the target, especially for mRNA with long 3'UTR (>1300 nt). However, for a distance between the miRNA site and the stop codon shorter than 15 nucleotides a severe drop in miRNA efficiency is expected. Proximity to the stop codon and to the polyA tail of the miRNA site increases the accessibility of the miRISC. Moreover, the binding of several different miRNAs to the 3'UTR promotes the cooperative repressing phenomenon (94). All these structural features are summarized in Figure 10.

Besides the above-mentioned canonical mechanisms of miRNA gene regulation through 3'UTR interactions, other “non-canonical” miRNA-mediated mechanisms of mRNA expression modulation have been reported. Some miRNAs have been shown to bind to the open reading frame (95) or to the 5'UTR of the target genes (96).

3.4.2.2. Categories of target sites

Several categories of target sites have been described according to their structural features (91, 97).

Table 1. Biocomputational tools for prediction of mRNA target sites in metazoans

Biocomputational tools	Criteria	Websites	References
Targetscan	A ¹ ; D ⁴ ; E ⁵ ; F ⁶ ; G ⁷	http://www.targetscan.org	(88, 98)
TargetscanS	A ¹ ; B ² (6-nt pairing necessary); D ⁴ ; F ⁶ ; G ⁷	http://www.targetscan.org	(91)
PicTar	A ¹ ; C ³ ; D ⁴ ; E ⁵	http://www.pictar.mdc-berlin.de	(99)
MiRanda	A ¹ ; B ² ; D ⁴ ; E ⁵	http://www.microrna.org	(100, 101)
Microcosm targets	Based on MiRanda software	http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/	(14)
PITA Top	A ¹ ; B ² ; D ⁴ ; E ⁵ ; G ⁷ ; H ⁸	http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html	(102)
EIMMo	A ¹ ; D ⁴ ; E ⁵	http://www.mirz.unibas.ch/EIMMo2	(103)
Diana-microT	A ¹ ; B ² ; C ³ ; D ⁴ ; F ⁶	http://diana.cslab.ece.ntua.gr/microT	(104, 105)

¹Strong pairing in the seed region, ²Moderate pairing in the seed region, ³Thermodynamic stability, ⁴Interspecies preservation of target sites, ⁵Number of target sites on the 3'UTR of a given mRNA, ⁶Sequence context (location of the miRNA sites with regard to the stop codon and to the polyA tail or presence of AU-rich clusters), ⁷Accessibility of the target site to the RISC complex, ⁸Possibility of tuning the search parameters

There are two main categories, which can be called canonical or marginal sites, and more rarely, atypical sites (Figure 11). Canonical sites are characterized by strong pairing in the seed region and contain three types of sites (8mer, 7mer-m8, and 7mer-A1). The 8mer sites have an interaction on 8 nucleotides including the 6 seed region nucleotides (nucleotides 2–7), and nucleotides in positions 1 and 8. The 7mer-m8 and 7mer-A1 sites include the 6 seed region nucleotides and, respectively, any nucleotide in position 8 (m8) or the A residue in position 1. Marginal sites are characterized by a weaker pairing in the 5' end of the miRNA, with pairing of only 6 nucleotides located inside the seed region (6mer) or located between nucleotides 3 and 8 (6mer offset). Finally, atypical sites are characterized by an increased number of complementary nucleotides at the 3' end.

3.4.2.3. Biocomputational tools for the prediction of target sites

Several tools and databases have been set up to assist in the identification of the mRNAs that are putatively targeted by miRNAs in mammals. Target identification is based on different criteria such as the binding duplex stringency characterized by the length of the pairing region and the thermodynamic stability of this binding, the conservation of the target sites between species, or the number of miRNAs targeting a specific 3'UTR part. The main biocomputational tools are referenced in Table 1.

TargetScanS is considered the simplified and improved version of TargetScan. Indeed, in this version, the necessary pairing is only 6 nucleotides in the seed region and the target site number on a single mRNA is not required in this version. The three biocomputational tools, TargetScanS, PicTar, and EIMMo have many common putative target sites because the engines look for sites with strong base-pairing in the seed region. However, there is no perfect overlapping between the results obtained with these three engines that use different UTR databases and/or miRNA sequence files (105). Moreover, these algorithms have some intrinsic differences in the criteria for mRNA target prediction. For example, TargetScanS requires that the mRNA target site ends by an A residue located in complementarity to the first miRNA 5' nucleotide, whereas the two other algorithms allow any Watson-Crick base pairing in this position (97).

The reliability of these algorithms is sometimes questionable (see for example (106)). In order to reduce

false-positive or false-negative targets of miRNAs, a useful tool, TargetCombo (<http://www.diana.pcbi.upenn.edu/cgi-bin/TargetCombo.cgi>), has been developed to combine many data sets. In particular it is possible to query files that combine all the targets predicted by at least one of the databases, Diana-microT, TargetScanS, miRanda and PicTar, or the targets all predicted by TargetScanS, miRanda and PicTar, or finally the results obtained with TarBase (105). The biocomputational tool TarBase (<http://diana.cslab.ece.ntua.gr/tarbase>) compiles all the experimentally validated targets (107, 108).

3.4.3. Molecular mechanisms of translation regulation of mRNA targets by miRNAs

3.4.3.1. mRNA maturation, stability, and translation: basic features

After transcription, mRNAs are processed by addition of a cap m7G at the 5' end, a polyA extension at the 3' end, and by appropriate exon splicing. Cap and polyA tail strengthen mRNA stability since they interact with specific protein complexes that prevent exonuclease activity due to steric hindrance. mRNA stability is also regulated by the AU-rich elements binding proteins (ARE-BP), which bind to AU-rich domains located in the 3'UTR of mRNAs.

In the cytoplasm, mRNA translation requires three major steps: initiation, elongation, and termination (109, 110). The initiation occurs at the level of the cap, with first association of the 40S ribosomal subunit, methionine initiator tRNA, protein factors to aggregate in a 43S preinitiation complex, and involvement of the eIF4F complex. The 43S complex then shifts to the initiation codon AUG and partially dissociates, while a 80S is assembled by binding the 60S ribosomal subunit prior to translation.

Alternatively, translation initiation may rely on the recruitment of ribosomes by an internal ribosome entry site (IRES) and does not need the loading of the initiation protein complex at the mRNA cap structure (111). The IRES sequences are characterized by stem-loop secondary structures in the mRNA 5'UTR that contain an initiation codon at their 3' ends. The 40S ribosomal subunit binds directly to the initiation codon and initiates translation. After translation initiation, the ribosome moves codon by codon and recruits amino acid-loaded tRNAs. At the level of a stop codon, the action of termination factors promotes

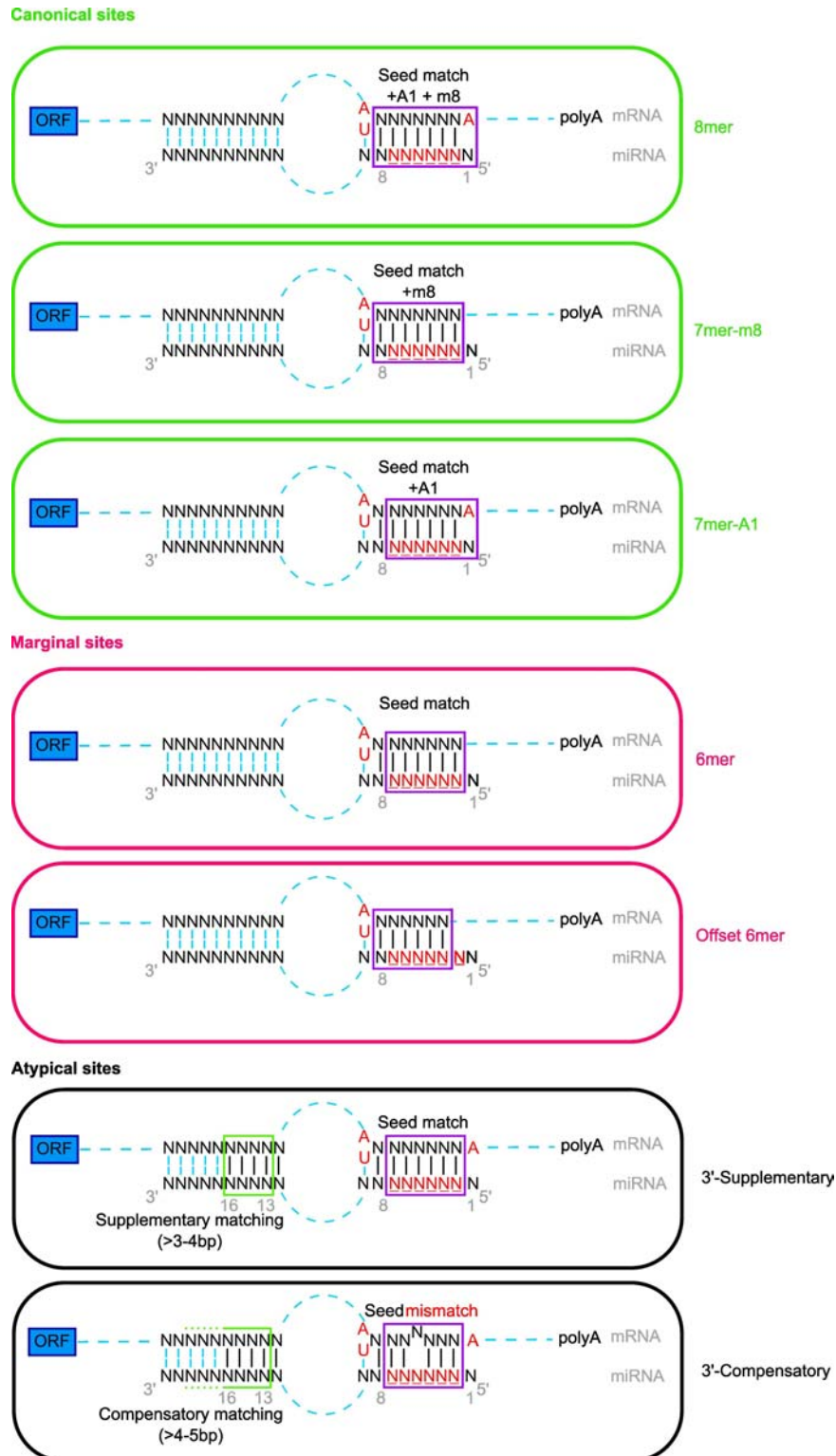


Figure 11. The three different categories of target sites. Canonical, marginal, and atypical sites (97). Canonical sites are composed of complementary sites of 7 or 8 nucleotides at the miRNA 5' end (6 seed region nucleotides + position 1 and/or 8). Marginal sites encompass complementarity sites for only 6 nucleotides in the seed region or 6 nucleotides between positions 3 and 8). Atypical sites show strand complementarity at the 3' end of miRNA to compensate for weak base-pairing in the 5' region. m8. nucleotide in position 8 A1. nucleotide A in position 1.

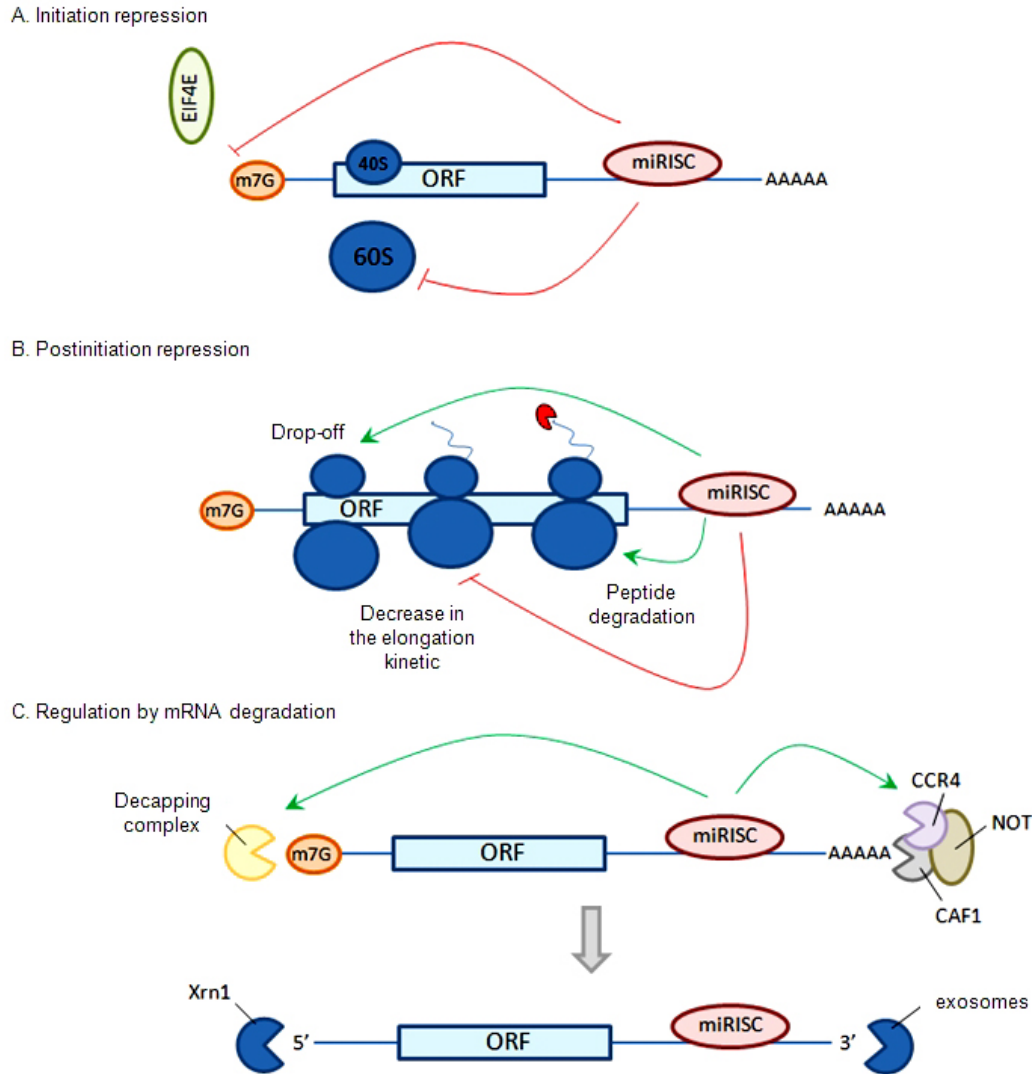


Figure 12. Mechanisms of regulation of mRNA target translation by miRNA. (A) Regulation by translation initiation repression. This regulation can occur either by inhibition of eIF4E fixation at the cap because of a competition with Ago proteins or by inhibition of the recruitment of the 60S ribosomal subunit. (B) Regulation by repression at the translation postinitiation step. Three mechanisms have been described. Regulation by premature release of ribosomes (drop-off), by decrease in the elongation kinetics, or by degradation of the newly synthesized peptide. (C) Regulation by mRNA degradation. A complex containing two deadenylases (CCR4 and CAF1) and NOT protein degrades the polyA tail. A decapping complex removes the cap at the 5' end and mRNA degradation can take place from 5' to 3' with the endonuclease Xrn1 or from 3' to 5' with exosomes.

ribosome dissociation and translation stops. Translation initiation is a key step for translation regulation (110), and it is frequently repressed by miRNAs.

3.4.3.2. Translation initiation repression by miRNAs

Several studies carried out on cultured cells demonstrated that the functional m7G cap is required for translation inhibition by miRNAs to take place. Indeed, miRNA inhibition of translation is impeded when mRNAs have a nonfunctional cap structure or when they are translated from IRES (112, 113). Some mRNAs have a bicistronic feature, with one cistron translated in a cap-dependent way and another one translated from IRES. In this case, only the translation of the first cistron is repressed by miRNA (113). The importance of the m7G cap structure in the regulation of

translation initiation is also illustrated by the fact that the central domain sequences of Ago proteins share some similarities with the central domain of the eIF4E factor, the cap-associated factor essential for translation initiation. In the Ago2 central domain, there are two phenylalanine residues that have proved to be necessary for binding Ago2 at the cap. Consequently, these proteins may inhibit initiation translation by competition with cap-associated factors such as eIF4E for binding to the cap (112-114). In addition, translation initiation repression mediated by miRNA may rely on interaction between the miRISC complex and the eIF6 factor. This factor prevents the association between the 60S ribosomal subunit and the 40S ribosomal subunit and blocks translation initiation (115). The different mechanisms of the regulation of mRNA target expression by miRNAs are shown in Figure 12.

3.4.3.3. Repression of the postinitiation step of translation by miRNAs

Studies conducted on *C. elegans* highlighted that lin-14 and lin-28 mRNAs, the targets of the lin-4 miRNA, are associated with polysomes, while they are poorly translated into proteins (116, 117). mRNA association with polysomes is usually a sign of strong translation activity. A low level of lin-14 and lin-28 mRNA translation could then be taken as an indication that miRNAs can play a role in translation inhibition at a postinitiation step. Similar situations were also observed in mammals. Indeed, Petersen et al. (118) reported that IRES-dependent translation of mRNA (a process that does not rely on a cap-dependent initiation step) can also be repressed by miRNAs. They found that miRNAs and their mRNA targets are present in translation-active polysomes and, in the presence of puromycin, which aborts the translation elongation step, miRNAs and mRNAs are finally found in the translation-inactive polysomes. It was then concluded that the postinitiation repression mechanism is due to a premature release of ribosomes (called drop off) during the elongation step (118). Postinitiation repression was also observed in HeLa cells in Kras mRNA, a let-7 target (119). Let-7 leads to inhibition of Kras expression and both the miRNA and its mRNA target were found associated with translation-active polysomes. However, the authors suggested that in this case, the miRNA-mediated postinitiation repression mechanism stems from a decrease in the speed of translational elongation. Finally, Nottrott et al. (120) also found miRNAs and mRNAs associated in polysomes, and concluded that translation repression by miRNA could be the result of a degradation of the newly synthesized polypeptide.

3.4.3.4. Regulation by degradation of the mRNA targets

Several studies reported that miRNAs repress protein expression with limited effects on the levels of the target mRNAs. Nevertheless, other studies reported a miRNA-mediated decrease in stability of the mRNAs. For example, in *C. elegans* let-7 decreases lin-41 mRNA stability and lin-4 reduces the stability of both lin-14 and lin-28 mRNAs (121). In human, lin-28 mRNA stability is lowered by miR-125a and miR-125b (122). Moreover, in mammals miRNAs were reported to reduce protein output mainly by destabilization of target mRNAs (123). Accelerated deadenylation of the polyA-tailed target mRNA was reported to be a key step in miRNA-mediated mRNA decay (124).

In eucaryotes, deadenylation is first initiated by the PARN2/PARN3 complex and then requires a protein complex composed of NOT and two deadenylases, CAF1 and CCR4 (125). Once deadenylated, mRNAs can be degraded by exonucleases from their two ends. The 5'-to-3' end degradation is performed by the Xrn1 exonuclease after cap removal. In human, this step involves a complex made of DCP2 and several cofactors such as DCP1, EDC3, Ge-1, and RCK/p54 (126). All these proteins involved in the 5'-to-3' end degradation are located in cytoplasmic granules called p-bodies (processing bodies) (125, 127, 128), while the 3'-to-5' end mRNA degradation is carried out by protein complexes called exosomes (129).

These p-body cytoplasmic granules, also important in RNA interference phenomena, contain the GW182 protein, a 182-kDa protein containing glycine-tryptophane (GW) repeated elements (130). This protein is able to bind to Argonaute proteins, present in p-bodies associated with miRNAs and mRNA targets (131). Stress granules are another kind of cytoplasmic granule produced by eucaryote cells subjected to stress, such as oxidative stress, thermic shock, or UV irradiation (132). Using immunofluorescent techniques, stress granules and p-bodies were proved to be two independent and distinct structures (133, 134). These cytoplasmic granules are a storage place for nontranslated mRNAs.

3.4.4. Translation activation by miRNAs

In most of cases, miRNAs play a role in negative regulation of gene expression by mRNA target translation repression or by mRNA degradation. Nevertheless, in a few cases positive regulation by translation activation has been observed and especially in conditions of cellular stress. ARE sequences (AU-rich elements) located in the mRNA 3'UTR are critical elements for translation activation. It was shown that Ago2 and FXR1 bind to ARE sequences in order to activate the translation of the TNF-alpha factor in cultured cells under serum privation and to stop the cell cycle (135). miR-369 which has a seed region complementary to two target sites of the ARE sequence of the TNF-alpha coding mRNA was shown to recruit Ago2 and FXR1 and to activate TNF-alpha translation under serum privation conditions. It has been concluded that miRNAs may play a role of translation repressors in proliferating cells and a role of translation activators in cells blocked at the G0/G1 stage. Consequently, Ago2 may be involved in either activation or repression of mRNA translation (135).

Interestingly, mRNA translation activation by miRNA was also reported to proceed through an indirect action of miRNA by Eiring *et al.* (136). In the specific case of the chronic myelogenous leukemia, miR-328 was found acting as a decoy by binding to heterogeneous ribonucleoprotein hnRNP E2 in a miRNA's seed sequence independent way and this relieves the hnRNP E2 dependent translational repression of the CEBPA mRNA involved in myeloid cell differentiation.

miRNAs play an important role in translation regulation since it is assumed that up to 60% of the protein coding genes can be under the control of miRNAs (98). They are strongly involved in many cell processes such as development, differentiation, proliferation, and apoptosis (21, 137-140), all processes that are often deregulated in tumors. miRNA are believed to provide major contributions in tumorigenesis and description of these pathological roles at a molecular level are now increasingly documented.

4. miRNAs IN TUMORIGENESIS AND TUMOR DEVELOPMENT PROCESSES

4.1. Modifications of miRNA expression levels in cancer

4.1.1. miRNA expression profiles in tumors

Different techniques can be used to assess the miRNA expression level in tumor samples such as

Northern blot analysis, quantitative PCR, oligonucleotide microarrays, SAGE and RAKE techniques, and quantitative flow cytometry or Next Generation Sequencing. Most of these techniques are useful for large-scale studies, involving large numbers of samples to be analyzed, or large numbers of different miRNAs to assay, or both.

Two of the early large-scale studies assayed many miRNAs in several types of tumors, using a quantitative flow cytometry technique based on the use of beads (141) or a microarray technique and Northern blots (142). Approximately 25% of the miRNAs studied were found to be significantly deregulated in at least one tumor type. The first study mainly revealed that miRNAs are underexpressed in tumor tissues in comparison to normal tissues, whereas the second one uncovered that miRNAs are overexpressed in tumor tissues. The different conclusions reported by these two studies conducted on similar types of tumor tissue (breast, colon, lung, pancreas, prostate, and stomach) probably resulted from the use of two different technical platforms or the sample size (142).

miRNA expression deregulation in several types of cancer were reassessed by taking into account the two above-mentioned original studies and the analysis of only one type of tumor (143). This highlighted that a number of miRNAs such as miR-21, miR-155, miR-221, and miR-222 appeared to be consistently overexpressed in many tumor tissues (brain, thyroid, gastrointestinal tract, liver, lung, and breast), whereas others such as miR-143, miR-145, and the let-7/miR-98 cluster were found to be underexpressed in tumor tissues. However, the expression of several miRNAs is not always modified identically in all types of cancer. For example, the miR-17/92 cluster has been reported to be overexpressed in seven tumor types and underexpressed in three others.

miRNAs are also interesting diagnostic biomarkers distinguishing different subtypes of tumors from the same tissue, such as in gliomas (144), thyroid cancer (145) or renal cell carcinoma (146).

4.1.2. Deregulation of miRNA expression by defects in the biogenesis mechanism

Altered expression of the proteins involved in the miRNA biogenesis and maturation machinery has been observed in several cancers. For example, deregulation of Drosha expression is associated with ovarian cancer (147), skin epithelium cancer (148), and esophagus tumors (149). A high Drosha expression level is associated with a poor survival prognosis. Interestingly, when esophagus tumor cell lines were transfected by siRNAs designed to target the Drosha coding mRNA, a significant decrease in the cell proliferation was noted (149).

Inactivating mutations in XPO5, coding for Exportin 5, have been discovered in several cancer cell lines with microsatellite instability (150). These mutations lead to trapping pre-miRNAs in the nucleus, thus preventing miRNA processing.

Dicer was also found to be overexpressed in lung carcinoma and prostate adenocarcinoma (151, 152) and underexpressed in high-grade ovarian carcinoma, small-cell lung tumors with poor prognosis, and invasive lung adenocarcinoma (147, 152, 153). In addition, frameshift mutations in the TRBP2 genes were found to diminish the TRBP protein expression. This causes destabilization of the DICER complex and a defect in the processing of miRNAs (154).

Expression and activity of some major components of the miRNA processing pathway such as Drosha/DGCR8, Dicer/TRBP2 or Argonautes may be regulated by the transcription factors p53, p63, and p73 (155). The promoters of the genes coding for Drosha, DGCR8, Dicer and TRBP contain p53-RE sequences indicating that they could be direct transcriptional targets of p53 and preferentially of p63/p73. Moreover, direct interactions between WW domains in p63 and p73 and PY domains in DGCR8 protein may influence miRNA processing. In addition, the pro-apoptotic TA-p63 isoform may indirectly decrease the expression of Dicer, for example, by increasing the expression of let-7, a miRNA that has been reported to reduce the expression of Dicer. Drosha, DGCR8, Dicer, and TRBP are also controlled by several miRNAs whose expression is regulated by p53. This suggests that p53/p63/p73 could regulate the expression of the miRNA processing components, at both the transcriptional and post-transcriptional levels.

4.1.3. Deregulation of miRNA expression by chromosomal rearrangements

Two studies highlighted that miRNA genes are located in chromosomal regions that are frequently altered in cancers (156, 157). Alterations of the copy number for 37%, 73%, and 86% of 283 miRNA coding genes were pinpointed in ovarian cancer, breast cancer, and melanoma, respectively. Nearly 40 miRNA coding genes are altered in common in these three types of tumor (157). Modifications of the expression of miRNA coding genes may result from chromosomal translocation, leading, for example, to overexpression of the gene coding for miR-125b, due to translocation t(2;11) in some types of leukemia (158). They may also be linked to amplification or deletion of genomic regions. This is exemplified in the 17q23 region, which was found to be amplified in neuroblastoma (159). This region hosts the gene coding for miR-21, which is usually upregulated in tumors. Another example is the presence of a deletion in the 13q14 region in several cases of B-cell chronic lymphoid leukemia. Genes coding for miR-15a and miR-16-1, underexpressed in 68% of these types of tumor, are located in this region (160).

4.1.4. Deregulation of miRNA expression due to point mutations in coding genes

The number of point mutations or SNPs (single nucleotide polymorphisms) in human pri-miRNA or pre-miRNA sequences has been reported to be usually very low (161). Nevertheless, several studies have indicated that SNPs may explain changes in miRNA expression between tumor and normal tissues. One SNP (G:C) in the pre-miR-146a sequence (60 nucleotides from the first nucleotide)

has been revealed and associated with a decrease in the expression of mature miR-146a in thyroid papillary carcinoma (162) and in prostate cancer (163). Moreover, a G/C heterozygous status in tissues is associated with a higher risk of thyroid papillary carcinoma in comparison to G/G or C/C homozygous status (162). In prostate cancer, a C/C homozygous status was reported to correlate to halving the risk of cancer in comparison to G/G or G/C genotypes (163). Another SNP (G:U), identified in the seed region of miR-125a located 8 nt from the 5' end, could have an impact on the interaction between miRNA-125a and its mRNA target (164). A G/U heterozygous status was observed in some cases of breast cancer (165). In non-small-cell lung cancer, an SNP (U:C) in the pre-miR-196a-2 (78 nucleotides from the first nucleotide) has been identified and patients with a C/C homozygous status had a shorter survival rate compared to patients with a U/U or U/C genotype (166). Calin *et al.* highlighted mutations in five out of 42 miRNAs in 15% of chronic lymphocytic leukemia (CLL) samples (167). In another type of lymphocytic leukemia, MLL-AF4 acute lymphocytic leukemia, miR-128b has been found to be underexpressed in tumor samples, due to a mutation in the primary transcript located 13 bp from the 3' end of mature miR-128b (168). This mutation also contributes to glucocorticoid resistance of tumor cells.

4.1.5. Modifications of miRNA expression due to transcriptional regulation

Transcription of miRNA encoding genes by RNA polymerase II can be regulated by several transcriptional factors. In the flanking regions of miRNA-coding genes in *C. elegans* and in humans the presence of patterns involved in transcriptional regulation has been studied (169). A number of transcriptional factors such as p53, E2F, and Myc have been reported to play a role in this regulation.

p53, known to be a tumor suppressor, has been highlighted as a transcriptional regulator of several miRNAs (170). It acts as a transcription activator of the miR-34a gene, as it can bind to the promoter region of this gene (171-173). It may also impair the expression of several miRNAs by inhibition of the transcription factor E2F1 (174). Activation by transcription factors belonging to the E2F family has been reported to increase the expression of some miRNAs (175). Another transcription factor, Myc, which has oncogenic properties, has been described as an expression modulator for a number of miRNAs. Indeed, by binding to promoter regions, Myc has been shown to be an expression activator of the miR-17/92 cluster, for example (176), but also an expression repressor for many miRNAs involved in proliferation processes (177).

Transcription regulation, which can account for a modulation of miRNA expression levels, can also result from epigenetic modifications at the promoter regions of specific miRNA coding genes. One important epigenetic modification is DNA methylation, which is a reversible phenomenon and involves only cytosines belonging to CpG dinucleotide motifs. CpG-rich genomic domains,

called CpG islands, can be located in several promoter regions. DNA methylation of CpG islands in promoters is a phenomenon that controls the expression level of downstream genes, as this modifies the chromatin structure and consequently the binding of transcription factors; in addition it favors MBD (methyl-DNA binding) protein fixation (178).

The role of DNA methylation in repression of miRNA expression was confirmed by using DNA demethylation drugs on cultured tumor cells that promote miRNA overexpression (179-181). Several miRNAs have been studied more specifically, such as miR-124 (181) and miR-34a (182), for which the lower expression in cell lines or in tumor tissues is due to a hypermethylation of CpG islands, and miR-128 (183) and let-7a-3 (184), which are overexpressed due to a hypomethylation of CpG islands.

4.2. Modifications of the interactions between miRNAs and their mRNA targets

4.2.1. Deregulation by chromosomal modification

As mentioned above, several genomic alterations, such as chromosomal rearrangements or point mutations, may rationalize the differences observed in miRNA expression between tumor and normal tissues. These alterations are important events in the tumor genesis process, as they prevent proper regulation of normal cellular processes by miRNAs. In addition, and similarly, genomic alterations can also affect the 3'UTR sequences of mRNA targets and lead to gene regulation modifications by impairment of the miRNA/mRNA target hybridizations.

One study demonstrated that a 4-nt TTCA insertion in the 3'UTR of the IL-1 alpha mRNA significantly disrupts a binding site for miR-122 and miR-378 and has a substantial influence on IL-1 alpha expression regulation (185). Studies conducted on mantle cell lymphoma have shown high expression of CCND1 in these tumors due to deletions and mutations in the CCND1 mRNA 3'UTR (186). These sequence modifications impair the interaction between CCND1 mRNA and miR-16, increasing the expression of the CCND1 protein (187). Genomic translocation can delete the normal 3'UTR part of the mRNA coding for HMGA2, making let-7 unable to bind to this region and to restrain HMGA2 expression (188). This protein has been shown to be involved in the tumorigenesis of many malignant tumors such as lung cancer (189-191) and pancreas cancer (192).

In the case of ABCG2 (a ubiquitous ATP-binding cassette (ABC) transporter, playing a role in absorption, distribution, and elimination of drugs), a shortening of the 3'UTR mRNA has been detected in drug-resistant colon cancer cell lines, leading to a loss of a miR-519c binding site. This relieves the repression of ABCG2 translation, contributes to the overexpression of the transporter, and confers drug resistance to the tumor cells (193).

4.2.2. Deregulation by point mutations

Yu *et al.* (194) investigated SNPs in the mRNA 3'UTR sequence complementary to several miRNAs. They noted fewer SNPs in the regions that are complementary to

the miRNA seed region than in the other part of the 3'UTRs (194). This was taken as evidence of a selection pressure and the importance of the regulatory mechanism by miRNAs.

SNPs located in miRNA complementary regions have been studied in a tumor context in an increasing number of reports. Two SNPs in the 3'UTR of the KIT mRNA (tyrosine kinase receptor) in thyroid papillary carcinoma have been described. These SNPs (G:A and G:C) affect the binding of miR-221/222 and miR-146a/146b miRNAs to the KIT mRNA, favor expression of the protein, and consequently increase the risk of cancer (195). In CD86 mRNA 3'UTR, a (G:C) SNP was found associated with a higher risk of sporadic colorectal cancer. This site is the target of five miRNAs; three experience a decrease in their binding affinity with the mRNA, contrary to the other two (196). Another study was conducted on the mRNA 3'UTR region coding for KRAS in non-small-cell lung cancer in moderate smokers. This mRNA contains ten target sites in the 3'UTR for the miRNA let-7 family. The authors described only one SNP per target site and most of these SNPs are infrequently found in tumors (197). Only one SNP (U:G) was found in about 20% of tumors and therefore can be associated with a higher risk of developing these tumors, and this type of mutation leads to an increase in KRAS mRNA expression *in vitro* (198). Another study was conducted on breast cancer and most particularly on integrins, proteins involved in the control of cellular attachment to the extracellular matrix. SNPs were found in the 3'UTR of integrin coding mRNAs, in particular ITGB4. A strong correlation was found between the presence of the polymorphic allele and the negative status of hormonal receptors in breast cancer (199).

4.3. Oncogene and tumor suppressor miRNAs involved in molecular pathways of tumor development

miRNAs can be considered as oncogenes when an increase in their expression contributes to a malignant transformation of normal cells, or on the contrary, as tumor suppressors in the reciprocal. The status of oncogenes or tumor suppressors is generally given to miRNAs that have been found to be deregulated in many tumor types. Several miRNAs have been described as oncogenes (frequently called oncomiRs). A few specific examples are given hereafter.

miR-372/373 are two miRNAs that have been described as playing a role in the development of testicular germ cell tumors in presence of active wild-type p53 and oncogenic RAS (200). The polycistronic cluster miR17-92 also seems to have an oncogenic role such as in lung cancer and B-cell lymphoma (201, 202). Indeed, overexpression of this cluster seems to accelerate tumor development of B-cell lymphoma in mice expressing c-myc. miR-155 is also described as an oncogenic miRNA, as it is overexpressed in B-cell lymphoma (142, 203-206). Some miRNAs, such as miR-21, are also considered as oncogenes when they can repress the expression of tumor suppressor proteins. miR-21 is overexpressed in many tumor types and targets the mRNAs of tumor suppressors PDCD4 and TPM1 (143, 207-210). On the contrary, other

miRNAs have been described as tumor suppressors: let-7 (156, 189, 211-216) as well as miR-15a and miR-16-1 (160, 217-220). miR-34a is also considered a tumor suppressor repressing several oncogenic genes, such as c-Met in brain tumors (221) and uveal melanoma (222) or Bcl-2 in neuroblastoma (223).

For a more comprehensive list of oncogenic or tumor suppressor miRNAs, the reader is referred to the miR 2 Disease database (www.mir2disease.org (224)).

miRNAs involved in tumor development can control many key signalization pathways such as apoptosis, cellular proliferation, and angiogenesis.

4.3.1. Apoptosis and cellular proliferation processes

The apoptosis process can be activated *via* two pathways, the intrinsic and the extrinsic pathways (Figure 13). In the intrinsic one, several pro-apoptotic and anti-apoptotic members of the Bcl-2 family are involved and their actions are antagonized to regulate mitochondrial membrane permeability. The anti-apoptotic proteins Bcl-2 and Bcl-xL are often overexpressed in tumors (225-227) and prevent mitochondrial permeabilization by inhibition of the cytosolic pro-apoptotic Bax and Bak (228). After a cellular stress, expression of pro-apoptotic members of the Bcl-2 family such as Puma and Noxa is enhanced by p53-dependent transcriptional activation. Subsequent activation of Bax and Bak proteins then allow their migration to the mitochondrial membrane where they concur to increase the permeability of the mitochondrial membrane. This permeabilization is associated with a release of cytochrome *c* into the cytoplasm (229), which binds to the APAF-1 protein (apoptotic protease-activating factor-1) and to two procaspases-9 to form the apoptosome. Then this protein complex promotes the activation of caspase-9, an apoptosis-initiating caspase (230). Activated caspase-9 cleaves procaspase-3, -6, or -7. In normal conditions, activation of these caspases is inhibited by proteins called IAPs (inhibitor of apoptosis protein). IAPs are inhibited by Smac/DIABLO proteins that are released into the cytoplasm after membrane permeabilization of mitochondria (231, 232).

As for the extrinsic apoptotic pathway, it starts outside of the cell with an activation of the pro-apoptotic receptors at the surface of the cell by ligands such as CD95L (FAS-L). Ligand binding to the extracellular domain receptors promotes intracellular domain binding to the FADD protein (Fas-associated death domain) (233). Then this complex recruits procaspase-8 or -10 and activates it. Activated caspases-8 and -10 then participate in the downstream steps of the intrinsic apoptotic pathway as they activate activator caspase-3, -6, or -7 and can also cleave and activate the Bid protein. The truncated protein tBid allows the relocalization of Bax and Bak (234).

A number of miRNAs have been shown to be able to regulate the apoptotic pathways at different stages (235). Several proteins of the Bcl-2 family are regulated by miRNAs. The Bcl-2 mRNA is the target of miR-15a/16 and it was demonstrated that both miRNAs negatively

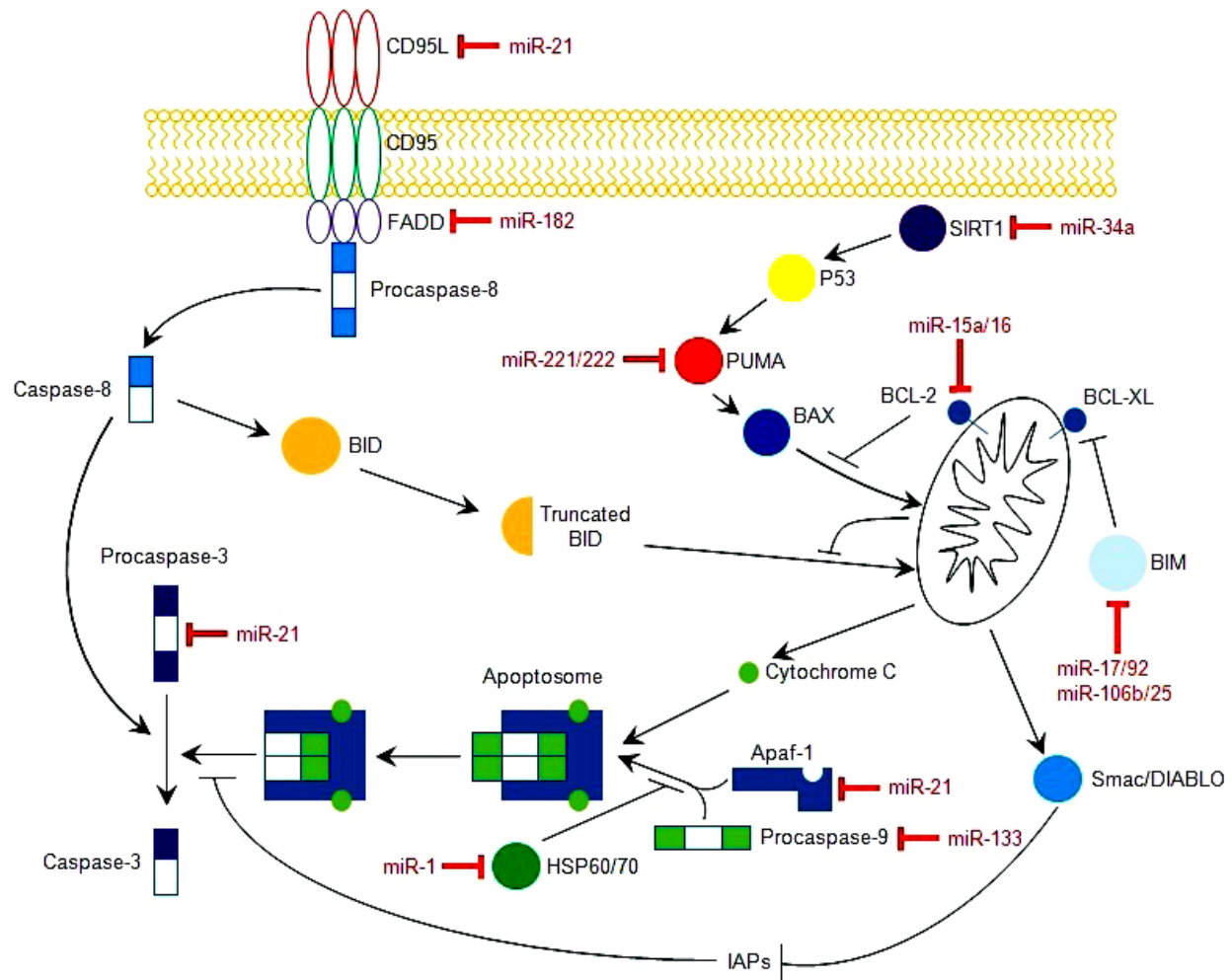


Figure 13. Implication of miRNAs in the apoptosis process (235). In the intrinsic pathway, the pro-apoptotic protein Puma is the target of miR-221/222 and is regulated by p53, target of the protein SIRT1, itself regulated by miR-34a. Anti-apoptotic protein Bcl-2 is the direct target of miR-15a/16, and Bcl-xL is the indirect target of some members of the miR-17/92 and miR-106b/25 clusters that regulate the pro-apoptotic protein Bim. Several miRNAs repress the apoptosome proteins, such as miR-21 targeting APAF-1 and miR-133 targeting caspase-9. miR-1 is a pro-apoptotic miRNA targeting HSP60 and HSP70, proteins that inhibit apoptosome formation. In the extrinsic pathway, concerning the CD95 receptor and its ligand CD95L (or FAS-L), miR-21 acts as a repressor of this ligand, whereas miR-182 lowers FADD expression. miR-21 also plays a role in caspase-3 regulation.

regulate Bcl-2 at a post-transcriptional level and induce apoptosis (236). The Bcl-xL protein is inhibited by the pro-apoptotic protein Bim whose expression level is downregulated by certain miRNAs of the miR-17/92 and miR-106b/25 clusters. Indeed, the miR-17/92 cluster is essential for B cell development, and absence of miR-17/92 leads to increased levels of the pro-apoptotic protein Bim and inhibits B cell development (237). miR-17/92 was found to be overexpressed in many cancers such as B-cell lymphoma. The miR-106b/25 cluster was found to be overexpressed in many gastric and prostate cancers. Some miRNAs, most particularly miR-106b, are assumed to target Bim but also p21, which is involved in cell cycle control, and E2F1 (238, 239). miR-34a is also an important regulator of the initiation of the apoptosis pathway, as this

miRNA lowers the expression of the deacetylase SIRT1. This leads to the acetylation of p53, the increase in its transcriptional activity, and the induction of the pro-apoptotic protein Puma (240). A recent study showed that Puma is the target of two miRNAs, miR-221/222, in glioblastoma cells (241). These two miRNAs also play an important role in cellular proliferation by regulation of p27^{Kip1} expression (242).

The second part of the intrinsic pathway, with apoptosome formation and activation of caspases, is also regulated by miRNAs. For example, miR-1 and miR-133 are co-transcribed from the same polycistron, but they have antagonistic roles in apoptosis, as miR-1 has a pro-apoptotic effect, whereas miR-133 is an anti-apoptotic

factor. In fact, miR-1 targets the anti-apoptotic proteins HSP60 and HSP70, two proteins that prevent apoptosome formation, while miR-133 represses caspase-9 expression (243). miR-21 also plays an anti-apoptotic role initially observed in glioblastoma cells (244), since this miRNA regulates the expression of APAF-1, caspase-3, and PDCD4 (245). In addition, PDCD4 also regulates caspase-3 activity (246).

The extrinsic pathway can be the target of miR-21 and miR-182, which control the expression of FAS-L (247) and protein FADD (248), respectively.

4.3.2. Angiogenesis process

Growth of new blood vessels from preexisting vessels, a process called angiogenesis, is a key step in tumor progression (249, 250). The control of this process by miRNAs has been evidenced to occur after the experimental knockdown of Dicer, by the transfection of specific siRNAs in endothelial cells. Indeed, the reduction of the expression of Dicer led to a decrease in angiogenesis, by a reduction of capillary sprouting of endothelial cells and tube-forming activity (251). After Dicer knockdown, Suarez *et al.* have shown an alteration in the expression of key genes in angiogenesis such as IL-8, angiopoietin receptor Tie-2, and VEGFR2 (252). Several studies analyzed the expression levels of miRNAs in endothelial cells and highlighted their pro-angiogenic properties *in vivo* (miR-126, miR-378, miR-296, and the miR-17/92 cluster), or *in vitro* (miR-210, let-7f, miR-27b, and miR-130a), or their anti-angiogenic properties (miR-221/222, miR-15b, and miR-16) (253). miR-126, the only endothelial cell-specific miRNA, was studied *in vivo* in a mouse model in which a drop in the expression of this miRNA was correlated with a decrease in angiogenesis and vascular integrity (254). The same conclusion was drawn in zebrafish in which knockdown of miR-126 leads to the loss of vascular integrity. This miRNA plays a role in the repression of negative regulators of the VEGF pathway, such as SPRED1 and PIK3R2, and it also seems that miR-126 regulates the expression of EGFL7 (255). This protein is also involved in many processes and particularly in angiogenic sprouting (256).

Other miRNAs, nonspecific of endothelial cells, such as miR-378, miR-296, and the miR-17/92 cluster, also have pro-angiogenic properties. The overexpression of miR-378 in glioblastoma U87 cells leads to angiogenesis of tumors after injection of the cells in nude mice by repression of the expression of tumor suppressors Fus-1 and SuFu (257). An inhibition of miR-296 in the glioblastoma U87 cells injected in nude mice reduces angiogenesis (258). Concerning the miR-17/92 cluster, overexpression in cells promotes the development of better-perfused tumors (259).

miR-210, a hypoxia-inducible miRNA, stimulates the cellular migration and the formation of vessel-like structures by downregulating Ephrin-A3 expression (260). miR-130a shows pro-angiogenic properties by targeting mRNAs coding for the anti-angiogenic proteins Gax and Hoxa5 (261). On the contrary, some miRNAs are more likely anti-angiogenic factors such

as miR-221/222, whose overexpression *in vitro* in endothelial cells leads to a decrease in cellular migration and the formation of vessel-like structures by repression of c-Kit (262). The miRNAs miR-15b and miR-16 target the VEGF-coding mRNA and thus have anti-angiogenic properties (263).

4.4. Exosomal miRNAs: new potential tumor biomarkers

Quantitative analyses of miRNA expression in tumor tissues revealed that miRNAs can be regarded as appropriate biomarkers that can help discriminate tumor types. miRNA profiling for tumor typing appears to be an attractive approach for diagnosis as a complement to pathology methods (144). This can be performed directly on tumor tissue specimens, but other less invasive approaches are currently under investigation. These approaches are based on the fact that miRNAs can be found in detectable amounts in small membrane vesicles (about 100 nm in diameter) called exosomes (264). Exosomes offer miRNAs a protection against RNases (265) and are secreted by a wide range of cell types such as hematopoietic cells, dendritic cells, mast cells, neurons, and tumor cells (266-268). Exosome purification and analysis of miRNA content may give new opportunities for diagnosis. In addition, exosomes and their RNase-protected miRNA are present in several biological fluids. Indeed, their presence has been evidenced in blood (268-272), urine (273, 274), saliva (275, 276) and amniotic fluid (277).

Assaying exosomal miRNAs in body fluids could be useful for cancer diagnosis. In fact, tumor-derived exosomes were proved to contain numerous tumor-specific miRNAs. For instance, exosomes can be enriched either in the let-7 miRNA family if derived from metastatic gastric cancer (278) or in various pre-miRNAs if derived from mesenchymal stem cells (279). miRNAs derived from glioma exosomes can therefore be envisioned for use as diagnosis biomarkers or surrogate markers for therapy follow-up (268, 269). For example, the exosomal miRNAs miR-21 and miR-155 extracted from blood have been reported to be valuable biomarkers for diagnosis of patients with lung adenocarcinomas (271). In ovarian cancer, exosomal circulating miRNAs can also be informative to distinguish various tumor stages (280). In prostate cancer cells, irradiation-induced premature senescence is associated with an increased release of exosomes. Thus, a quantitative assessment of the tumor exosomes released in body fluids should constitute an easy means for therapy follow-up (281).

It should be noted that exosomes may also have considerable implications in cancer development, given that these vesicles have been reported to be involved in an intercellular transfer of the mRNAs and miRNAs they contain between glioma cells (282), or from tumor cells into normal cells in the tissues (264, 283). Also, exosomes derived from cancer stem cells, which seem to be responsible for the increase in tumor growth, invasion, or vascularization, could establish a premetastatic niche away from the tumor tissue (284).

4.5. miRNAs in cancer therapy

With the discovery of the role of miRNAs in controlling the expression of many proteins, and moreover that they behave as oncogenic or tumor suppressor components, it soon became clear that miRNAs can be involved or may be used in new therapeutic approaches. It goes without saying that taking advantage of miRNAs in new antitumor developments requires that a number of hurdles be overcome (285).

Briefly, miRNAs intrinsically have a number of attractive features for therapy. Their mechanism of action guides the therapeutic process. In comparison to small inhibitory molecules that reduce the activity of targeted enzymes but leave the amount of cellular enzyme intact, miRNAs can be used to reduce the cellular concentration of targeted proteins. miRNAs are natural cellular components and they include an intrinsic signature that may ensure specificity to target. These may have important consequences in the control of side effects. Nevertheless, accurate miRNA-target recognition is not perfectly managed (see above) and off-target effects are still dreaded. On the other hand, one single miRNA can target several oncogenic proteins and the opportunity to simultaneously repress several oncogenes with only a single miRNA appears very attractive in the search to improve anticancer therapy, while limiting possible side effects. An example of this multitarget therapy can be found with miR-34a, which represses c-Met, CDK4 (cyclin-dependent kinase 4), and Bcl-2 (B-cell lymphoma 2), all involved in separate oncogenic pathways (286).

miRNA-based therapies can be tentatively classified according to two different criteria: the antitumoral effects that are strategically expected and the mode of action triggered. First and most specifically, several antitumoral effects can be putatively promoted using miRNA-based strategies (287). Indeed, given the numerous roles played by miRNAs in the tumor physiopathology mentioned above, modulation of miRNA expression in tumor tissues may have direct effects on several biochemical pathways and proliferation, survival, differentiation, and migration of tumor cells. Hence therapeutic strategies can include direct tumor suppressive effects, antiangiogenic effects, antimetastatic effects, suppression of immune evasion of tumors, or sensitization of tumor cells to traditional anticancer treatments such as radiotherapy or chemotherapy.

Second, miRNA-based therapies can be based on the development of miRNA mimicking compounds to increase the cellular concentration of any given miRNA or on the contrary of miRNA antagonists to reduce their level. Upregulation of miRNA cellular concentration can be obtained by transfection of the cells with exogenous miRNA, *i.e.*, pre-miRNA or mature miRNA, whereas antisense-mediated inhibition is based on the use of complementary antisense oligonucleotides of mature miRNAs to downregulate miRNAs (287). A third attractive therapeutic approach called indirect miRNA-based therapy takes advantage of the fact that some miRNAs are expressed in reduced amounts in cancer cells but are

present in high concentration in normal cells. The introduction of specific vectors or constructs containing a miRNA-regulated suicide gene in cancer cells may result in the expression of the given suicide gene only in the cancer cells that are devoid of the suicide gene-regulating miRNA. This strategy may be very safe and leave all normal cells unaffected.

miRNA replacement is the approach based on the administration of synthetic miRNAs so as to increase the cellular concentration of tumor suppressor miRNAs whose levels are frequently lowered in tumors. For example, in the case of let-7, which is a tumor suppressor downregulated in several cancer types, an intratumoral introduction of the let-7 oligonucleotide into a mouse model of non-small-cell lung cancer led to a reduction of the tumor size (288). Exogenous delivery of a miR-34 mimic by transfection or use of lentivirus constructs resulted in a reduction of the tumor in gastric cancer (289). miR-based strategies are under investigation at a preclinical stage for development of miRNA replacement therapies against cancers. For example, several are based on the delivery of mimics of miR-34 miR-16 or let-7, which are said to be tumor suppressors in tumors (Mirna Therapeutics, Austin, TX, USA) (286).

Antagonists of miRNAs are required to reduce the cellular levels of oncogenic miRNAs that are overexpressed in tumors. This can be achieved using antisense oligonucleotides (ASO) that can hybridize to miRNAs and then prevent miRNA interactions with the mRNA targets. Chemical modifications are needed to protect ASOs from nuclease degradation and to improve their affinity for miRNAs (290). The main modifications are the use of 2'-O-methyl-RNA nucleotides or locked nucleic acid (LNATM) nucleotides. In the locked nucleic acid nucleotides, the ribose ring is modified by a methylene bridge connecting the 2'-O atom and the 4'-C atom. A recent study has successfully used 2'-O-methyl- and DNA/LNA-mixed oligonucleotides to specifically knock down miR-21 and to investigate the potential contribution of this miRNA in the regulation of apoptosis-associated genes in glioblastoma cell lines (244). In these cell lines, Papagiannakopoulos *et al.* compared these two miR-21 downregulating types of ASOs and observed greater knockdown using LNA-complementary antisense miR-21 than the 2'-O-methyl-complementary antisense miR-21, increasing the levels of major components of the apoptotic pathway (245).

miR-122 is a miRNA expressed in liver that is essential for replication of the hepatitis C virus (HCV) RNA genome, causing liver disease and increased risk of hepatocellular carcinoma. Therapeutic silencing of miR-122 using a locked nucleic acid (LNA) -modified oligonucleotide (SPC3649) complementary antisense to miR-122 was proved to suppress viremia in primates (291). The antgomiR-122 (SPC3649 or Miravirsen - Santaris Pharma A/S, Hørsholm, Denmark) is currently being evaluated in Phase 2a clinical trials. Regulus Therapeutics (San Diego, CA, USA) is developing proprietary chemically modified anti-miR oligonucleotides delivered

systemically to target miR-21, which is overexpressed in many cancer types (142) and can promote tumor progression and metastasis (292). Regulus's anti-miR-21 downregulates miR-21 in the liver and reduces hepatocellular carcinoma (HCC).

Sensitization of tumors to chemotherapy or radiotherapy by miRNA-based therapy also seems to be a valuable approach according to a number of published results. For example, the oncogenic cluster miR-17/92 has been shown to be involved in the radioresistance of tumor cells. Indeed, overexpression of these clustered miRNAs in mantle cell lymphoma cells decreases the radiosensitivity of these cells by targeting PTEN and activating the PI3K/Akt signal pathway (293). In a similar way, miR-221/222 are also described as being involved in the radioresistance of tumor cells in gastric cancer. A knockdown of these miRNAs in cancer cell lines using complementary antisense oligonucleotides (2'-O-methyl-oligonucleotides) increases PTEN expression and increases the radiosensitivity of the tumoral cells (294).

The role played by miRNAs in the chemoresistance process ((295) for review) and as biomarkers of chemoresistance has been demonstrated. Ranade *et al.* found that miR-92*, miR-147, and miR-574-5p are overexpressed in small-cell lung cancer and are potential biomarkers of chemoresistance (296). In other types of cancer such as colon cancer or ovarian cancer, several other miRNAs have been described as being involved in chemoresistance. It has been shown that miR-140 and miR-215 are overexpressed in drug-resistant colorectal cancer cell lines and the targeted proteins involved in this resistance may be HDAC4 and DHFR, respectively (297, 298). In ovarian cancer, several miRNAs are involved in drug resistance, such as miR-30c, miR-130a, and miR-335, which are downregulated in drug-resistant cell lines and promote upregulation of M-CSF (macrophage colony-stimulating factor), a previously described resistance factor for ovarian cancers (299).

Collectively, all these studies show that appropriate control of specific miRNA concentrations in tumors may have beneficial impacts on tumor initiation and promotion. One main obstacle to these therapeutic strategies, as pointed by Purow (285), probably relates to the correct delivery of the miRNA mimics or antagonists to tumor cells.

5. CONCLUSION

miRNA appears to be an original class of noncoding RNAs. Approximately 1000 different miRNAs are expressed in human. This rather limited number may facilitate the understanding of their global physiological or pathological roles in cells. However, the large number of mRNAs that they can individually target makes the analysis extremely complicated. Moreover, additional factors or events probably remain to be identified to achieve a complete appraisal of the way mRNA expression is finely tuned in cells under the control of miRNAs.

Study of miRNAs in cancer obviously provides new opportunities for diagnosis. On the other hand, given their critical role in the control of gene expression and in their possible involvement in molecular dialogs between cells driven by exosomes, research programs in which miRNAs are regarded as targets for new therapeutic drugs or approaches seem perfectly warranted and look promising.

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Abbreviations: miRNA: microRNA; siRNAs: short interfering RNA; MiRISC: miRNA-induced silencing complex

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