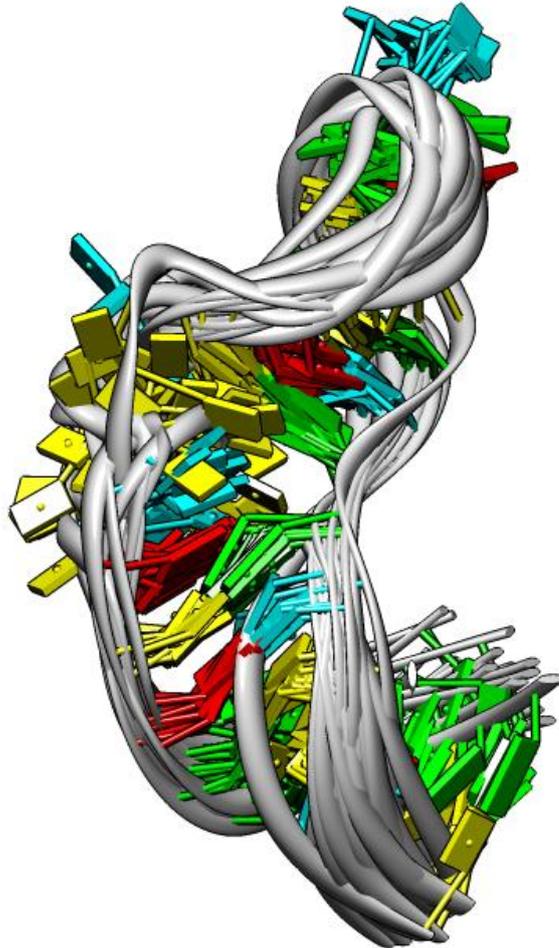


MicroRNAs in breast cancer



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**Master Thesis Biology of Disease
Faculty Biomedical Sciences**



Universiteit Utrecht

University of Utrecht, October 2010

Master Thesis Biology of Disease

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Introduction

A strict balance between cell growth, differentiation and apoptosis is necessary for normal tissue development. Many genes are involved to make sure that developmental processes succeed in a normal spatial and temporal fashion. Genes involved in these processes require appropriate transcriptional and post-transcriptional regulation. Up to 30% of all human protein-coding genes is thought to be post-transcriptionally regulated by small RNAs, called microRNAs (miRs).¹ These molecules are important in many physiological processes, including proliferation, differentiation, apoptosis and the maintenance of cell and tissue identity.^{2,3} If miRs or their regulatory factors do not work appropriately and disturb the above mentioned physiological processes, they may contribute to human pathologies, such as cancer.^{4,5} Indeed, recent research indicates that miRs are involved in various types of cancer, including breast cancer. Experiments in cell cultures and mouse models have revealed deregulated miRs that exert either tumor suppressive or oncogenic roles in cancer cells, depending on the cellular context and on the target genes they regulate.^{108,145} Altered miR expression has been linked to tumor initiation, progression and metastasis.^{303,306} However, the exact molecular mechanism of miRs and their role in tumorigenesis still needs to be elucidated. Based on computational algorithms, many miR target genes were predicted, but relatively few miR-target interactions have been experimentally validated. Modulating miR expression *in vitro* or *in vivo* may provide a powerful tool to investigate the molecular mechanisms and target genes regulated by these small RNAs. Such studies may provide exciting steps towards the potential usefulness of miRs in improving cancer diagnosis and therapy. In this paper, the working mechanism of miRs and their involvement in human breast cancer will be discussed.

Structural and functional features of microRNAs

Identification

Since the mid-nineties, a novel post-transcriptional molecule was identified by examining genes involved in developmental stages of the nematode *Caenorhabditis elegans*.⁶ To become adult roundworms, *C. elegans* pass through four different larval stages (L1 to L4) which are controlled by heterochronic genes. Cell lineages of *C. elegans* were mutated in order to study their effect on developmental patterns. It was found that in *lin-4* mutants, L1 specific cell-division patterns were repeated at later larval stages, suggesting an important role of *lin-4* in controlling the timing of developmental processes in this organism.⁷ Similar to *lin-4*, genetic mutations in *lin-14*, *lin-28* and *lin-29* also caused timing defects of post-embryonic developmental events in *C. elegans*.^{8,9} Interestingly, mutant *lin-14* showed opposite effects to developmental timing compared to mutant *lin-4*. *Lin-14* null mutations caused failure of L1-specific events and premature development into the L2 stage, while *lin-14* gain of function mutations resulted in an almost identical *lin-4* mutant phenotype.¹⁰ In agreement with this finding, *lin-4* mutants showed elevated levels of *lin-14* activity. This interaction and their opposite phenotypes suggest that they may negatively regulate each other. Further investigation of double mutant *C. elegans*, containing a *lin-4* mutant and a *lin-14* loss of function allele, showed only mutant *lin-14* associated defects, implicating that *lin-4* heterochronic defects require *lin-14* activity.⁸ Together, these results suggested that wild-type *lin-4* acts as a negative regulator of *lin-14*.

During normal development of *C. elegans*, a high level of *lin-14* protein is detected in the first larval stage (L1), while this gradient fades at later developmental phases (figure 1). However, *lin-14* transcripts are constant during all developmental stages, which indicates that *lin-14* is subjected to posttranscriptional regulation.¹¹ *Lin-14* gain of function mutations caused high *lin-14* protein levels at later larval stages. Mapping of the *lin-14* gain of function mutations revealed that these mutations are deleted sequences in *lin-14* mRNA 3'untranslated region (UTR), implicating the role of 3'UTR elements in mediating down-regulation of *lin-14* protein in late developmental phases.¹² For this reason, posttranscriptional regulation of *lin-14* by *lin-4* would probably also act via the *lin-14* 3'UTR. In agreement with this assumption, the *lin-14* 3'UTR was found to be necessary and sufficient for regulation by *lin-4*. Furthermore, conserved elements in *lin-14* 3'UTR were complementary to *lin-4* regulatory RNAs. These complementary sequences were required for the temporal decrease in *lin-14* protein level. Together, these results indicate that *lin-14* mRNA is post-transcriptionally inhibited by product(s) of *lin-4*.¹¹

Following the observation that *lin-4* post-transcriptionally regulates *lin-14* mRNA inhibition via *lin-14* 3'UTR, the group of Ambros cloned the *lin-4* locus to analyze *lin-4* genomic sequences. Interestingly, they found that *lin-4* encoded two small *lin-4* RNAs (instead of a protein), that were partially complementary to seven conserved sites located within the 3'UTR of *lin-14* mRNA.⁶

In conclusion, small RNAs can inhibit the translation of target mRNA by binding to complementary sequences at the 3' UTR of mRNA, thus controlling development.

This novel kind of natural antisense gene expression regulation has led to the discovery of a second *C. elegans* miR in the year 2000, *let-7*. *Let-7* encodes a 21-nucleotide RNA controlling developmental timing from larval stage 4 into adulthood. Similar to *lin-4* miR, *let-7* miR is complementary to elements in the 3'UTR of its target genes, *lin-41* and *hbl-1* (*lin-57*), and inhibits their translation.¹³⁻¹⁵

Now that two regulatory miRs were identified in *C. elegans*, it was questioned whether these short RNAs were also present in other species. Subsequent research identified homologues of *let-7*, *lin-4*, *lin-28* and *lin-41* in other vertebrates, including mice and humans, suggesting a more general role of miRs in regulating developmental processes.¹⁶⁻¹⁹

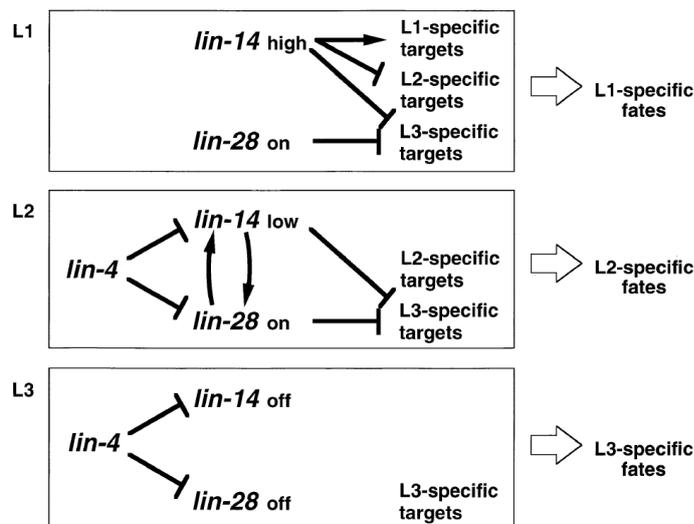


Figure 1. *Lin-4*, *lin-14* and *lin-28* expression during the first three developmental stages in *C. elegans*. High levels of *lin-14* are required for L1 specific events, while high *lin-14* levels inhibit L2 and L3 specific events. Activation of *lin-28* is also important for the regulation of L1 specific cell fate. *Lin-4* negatively regulates *lin-14* and *lin-28*, mediating their downregulation, but maintaining enough activity to induce L2 specific regulation. *Lin-14* and *lin-28* interact with each other, to support L2 specific cell fates, but the mechanism of action is unknown. L3 specific regulation occurs upon concomitant inhibition of *lin-14* and *lin-28* transcription.²⁰

Working mechanism

During the identification of *let-7*, another gene silencing-mechanism was discovered which resembled the miR mechanism of action. Fire and colleagues found that injection of (ds)RNA into *C. elegans* resulted in degradation of homologous mRNA, by a process termed RNA interference (RNAi).²¹ dsRNA was at least tenfold more potent in sequence-specific gene silencing in worms than were sense and antisense RNA alone.²¹ Thus in worms, gene silencing can be triggered by introducing dsRNA. In plants, silencing can be triggered by inverted homologous transgenic sequences, and transgenic plants were subsequently resistant to viruses. The notion emerged that sequence-specific gene silencing mechanisms in both plants and worms might share a common biological root. As mutations in RNAi components of plants resulted in higher sensitivity for viruses, RNAi was initially assumed to work as a natural cellular defense mechanism against exogenous dsRNA, including viruses, parasites and transposons.¹⁵ We understand now that RNAi is related to

a group of RNA-silencing phenomena which all have in common small RNAs that guide protein complexes to target genes or mRNA, to silence their expression. Within this group, miRs are included that repress multiple target genes to which they are partially complementary. Knowledge about the RNAi mechanism of action has provided important understanding of miR biochemistry. Actually, elucidating the biological mechanism of miRs started with the identification of RNAi and its mode of action.¹⁵

Processing of siRNA

To study the silencing mechanism of RNAi, a cell free lysate was prepared from *Drosophila melanogaster* syncytial blastoderm embryos. Subsequent addition of dsRNA together with 5'-capped target RNA, resulted in sequence-specific target RNA degradation.^{22,23} Furthermore, pre-incubation of dsRNA enhanced the degradation process, suggesting that covalent modification or specific factors in the lysate extract that associate with dsRNA, are necessary to convert dsRNA into an active form.^{22,24} Supportive to this assumption, both strands of the dsRNA in the lysate system were found to be processed into fragments of 21 to 23 nucleotides, which were necessary for target mRNA degradation.²⁴ Moreover, Elbashir *et al.* demonstrated that synthetic short RNA duplexes of 21 and 22 nucleotides induced sequence specific degradation when base paired with 3' overhang ends, which was necessary for efficient target cleaving.²³

The conversion from dsRNA to RNAi fragments was mediated by RNA polymerase-III enzymes (RNaseIII), consisting of two Dicer molecules that show specificity for dsRNA and generate 5' phosphorylated termini to the RNAi. Subsequent analyses of extracts from *Drosophila* cells transfected with dsRNA revealed that Dicer contained dsRNA fragments and triggered the assembly of a nuclease multicomplex, called RISC (RNA-induced silencing complex).²⁵⁻²⁷ siRNA needs ATP and the Argonaute protein 2 (which will be discussed later on) for unwinding of dsRNA. It was shown that a protein-siRNA complex containing an unwound siRNA strand, was required for RISC to become activated. Subsequently, RISC uses the unwound siRNA to target corresponding mRNA, which is then cleaved (figure 2).^{15,28,29}

Processing of miRNA

The mode of action to process longer dsRNAs into smaller fragments has also been shown in miR biogenesis, although siRNAs and miRs differ in their origin. siRNA originates from dsRNA and is primarily exogenous in origin, derived directly from viruses, transposons, or a transgene trigger.³⁰ However, siRNA may also be endogenous, which has been found in experiments in flies and mice.³¹⁻³³ MiR originates from single stranded RNA that forms a hairpin structure and is viewed as endogenous, because miRs are expressed products of an organism's own genome. Perhaps the clearest distinction between miRs and siRNAs is that siRNAs almost all silence the same locus from which they were derived, while the overwhelming number of miRs silences other genes than their own loci. Furthermore, in contrast to siRNAs, miRs have highly exact ends, which probably allow them to interact very specifically with substrate RNAs. siRNAs tend to have a much more heterogeneous end composition. These features make siRNAs so suitable to their role in defense, and explains why miRs are more severely constrained in the precision of their sequence structure.³⁰

Primary (pri)-miRs are long (generally >1 kb) transcriptional products of RNAse-II or are transcribed from independent miR genes. Some miRs can also be transcribed by RNAse-III.³⁴ A single pri-miR often contains sequences of different miRs and is characterized by an imperfectly base-paired hairpin. This hairpin is recognized by the nuclear RNAse-III enzyme Drosha, which contains double stranded RNA binding domains. Drosha together with a protein called Pasha in *Drosophila*, or DGCR8 (DiGeorge syndrome critical region gene 8) in mammals cleave the pri-miRs into a ~70 nucleotides long double stranded pre-RNA hairpin with 2 nucleotides overhang at the 3' end.³⁵ Upon recognition by exportin 5 and Ran-GTPase, the pre-miR is transported out of the nucleus into the cytoplasm (figure 2).^{36,37}

Despite earlier mentioned differences between miRs and siRNAs, these classes of small RNAs show similarities in size and sequence-specific inhibitory functions. Moreover, both classes depend on the same two protein families: Dicer and Argonaute.^{38,39}

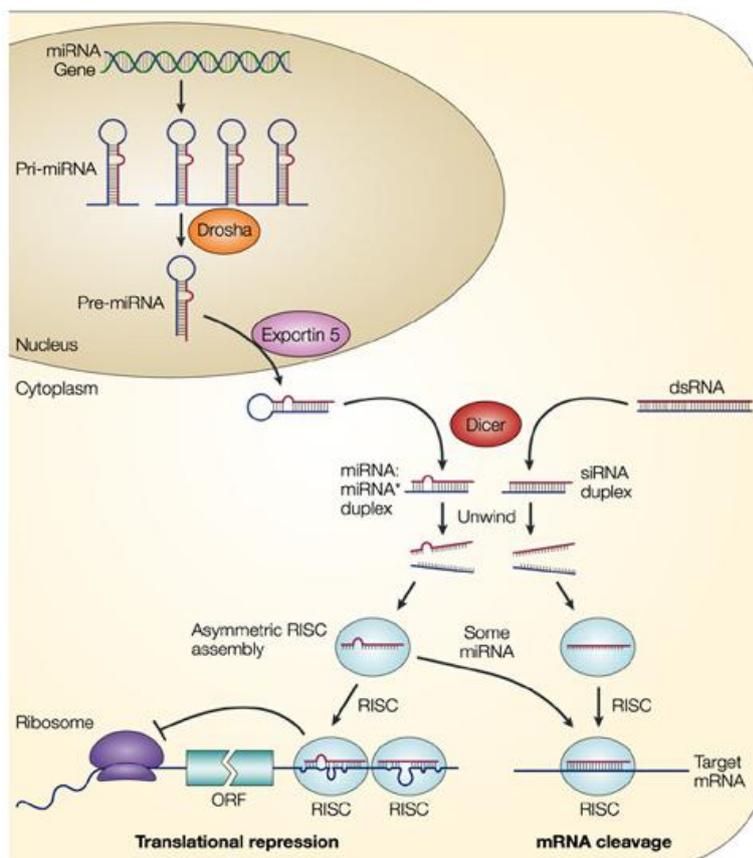


Figure 2. Processing siRNA and miR. Double-stranded pri-miR, usually consisting of imperfectly base-paired hairpin loops, is cleaved by RNA-polymerase Drosha into a ~70 nucleotides long pre-miR double strand. Pre-miR is transported from the nucleus into the cytoplasm by the Ran-GTP dependent transporter Exportin 5 and further cleaved into ~22 nucleotides short fragments. Exogenous dsRNA is also cleaved by Dicer into a siRNA duplex, which then follows the same downstream pathway as miR. Unwinding and RISC assembly is necessary to induce translational repression or mRNA cleavage. Translational repression can be achieved by binding imperfectly complementary to target mRNA, while mRNA cleavage results from perfectly complementary binding to target mRNA.⁴⁰

Dicer

Northern blot analyses of *C. elegans* revealed miRs of ~70 nucleotides as well as miRs of ~22 nucleotides in the cytoplasm, implicating that the longer RNAs may be precursors of the short RNAs.^{6,13} Because Dicer was shown to regulate dsRNA cleavage into ~22 nucleotides long fragments in RNAi, it was suggested that maturation of miR could also be regulated by Dicer. This enzyme family is evolutionarily conserved, including orthologs in humans that share the

characteristics of recognizing dsRNA and ATP-dependent cleavage of dsRNA into RNAi.^{28,41-44} Indeed, in mammals, *in vitro* synthesized pre-miRs were shown to be cleaved by Dicer. Subsequently, they form a complex with TAR RNA binding protein (TRBP) which binds to double stranded RNA. Double stranded RNA is processed by the TRBP-Dicer complex into mature miRs. This observation was supported by miR maturation analysis in knock-down and knock-out studies of Dicer homologues in *C. elegans* and mice, showing defects of RNAi.^{41,41,43,45} However, recent observations in genetically modified mice revealed a Dicer-independent maturing miR. This miR, miR-451, instead becomes loaded onto Argonaute (Ago, an endonucleolytic cleavage protein) whose catalytic centre is responsible for the cleavage process. Although no structural human homologues to miR-451 are found thus far, this observation may suggest that additional miRs rely on this alternative biogenesis pathway for maturation.⁴⁶ In general, the strand with the 5' terminus located at the thermodynamically less-stable end of the dsRNA, functions as a mature miR.⁴⁷ Similar to RNAi, the unwound ~22 nucleotide mature miR will then be incorporated into ribonucleoprotein complexes or RISC, while the other miR strand is degraded. Unwinding of miR seems to occur differentially from unwinding of siRNA, but the exact process is still mysterious.⁴⁸ Unwinding was originally thought to require an ATP-dependent helicase to separate two RNA strands. However, later evidence revealed that human Ago2 directly receives dsRNA from the RISC-complex and may be an ATP-independent process. MiR unwinding was promoted by mismatches in the seed- or 3'-mid regions of *Drosophila Melanogaster*.⁴⁹ This may also explain the ATP-independent unwinding of miR in humans, although the exact mechanism needs to be elucidated.

RISC and Argonaute

One of the most important and best-characterized components of the RISC complex is the Argonaute (Ago) protein family.^{29,50,51} One way of miRs (and siRNAs) to control gene expression is by inducing site-specific cleavage of target mRNA, which has first been shown by providing dsRNA exogenously to a cell free lysate, but was later also discovered to occur endogenously in plants and metazoans. This endonuclease cleavage activity is referred to as 'slicer activity'.^{22,52} It was shown that the cleavage process requires the catalytically active Argonaute protein in the RISC complex, and sufficient base-pairing with a perfect or near-perfect match to target RNA.^{52,53}

In humans, four Ago proteins are recognized to function in mRNA repression, but only Ago2 is capable of cleaving target mRNA.⁵⁴ All Ago proteins have four conserved characteristic domains: an N terminal, Mid (amino middle), PAZ (Piwi Argonaute Zwiller) and PIWI (P-element induced wimpy testis domain of *Drosophila Melanogaster*). Both Ago and Dicer family proteins contain a common PAZ domain.^{55,56} Structural analyses of Ago2 showed that the PAZ domain recognizes and binds the two nucleotides 3'overhang of a mature siRNA or miR strand for incorporation into RISC.^{56,57} This strand is the guide RNA strand for mRNA target recognition. The cleavage site in the mRNA is defined by the 5' end of the guide RNA strand.^{23,58} In particular, the site of cleavage appeared to be between the nucleotide positions 10 and 11 in the siRNA/miR:mRNA duplex.^{23,53,59} Structural analyses of the Argonaute PIWI domain revealed a conserved preformed hydrophobic pocket that contains the binding site of the 5' phosphate of the guide RNA, and was shown to

contain endonuclease activity. Subsequent studies indicated that the MID domain can also bind 5' phosphate residues of the guide RNA (figure 3).^{57,60,61}

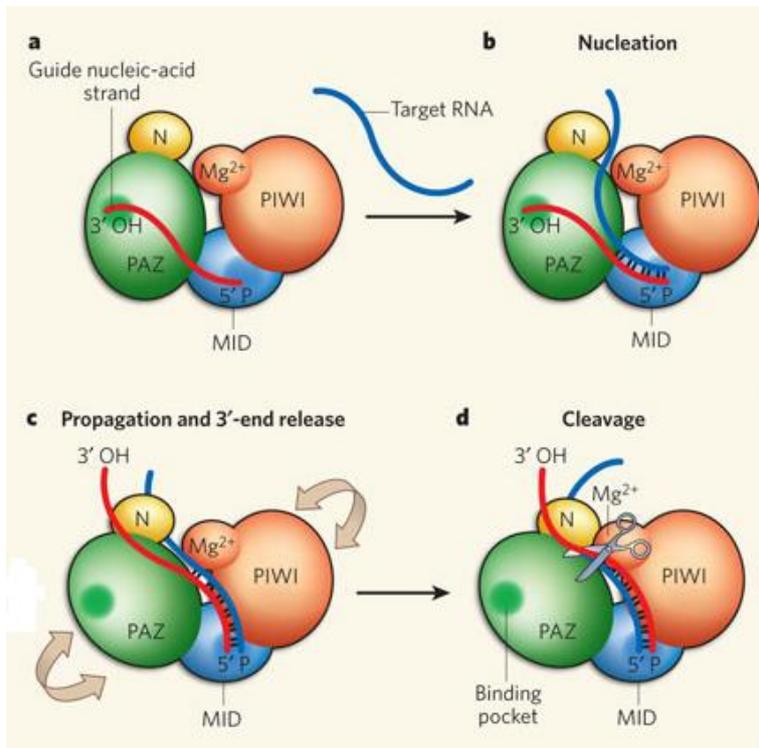


Figure 3. Argonaute proteins in the RNA-induced silencing complex. **a**, Argonaute proteins contain an amino-terminal (N), a PAZ, PIWI and MID domain. The 5' -phosphate end of an endonuclease cleaved siRNA or miR strand is bound to the MID domain, while the 3' end of the RNA containing the hydroxyl group will bind to the PAZ domain. **b**, The argonaute complex binds to target RNA which base-pairs to complementary sequences in the guide strand, starting at the 5' -phosphate end in the MID domain. **c**, Movement of the argonaute proteins releases the guide strand from the PAZ domain, allowing a conformational rotation. **d**, Exact positioning of the cleavage binding site allows cleavage of the helix by the PIWI domain, probably facilitated by magnesium ions.⁶²

However, not all PIWI domains within Argonaute proteins harbor a catalytic active Asp-Asp-His (DDH) motif, suggesting that additional factors are required for target cleavage.⁵⁴

So, miR cleavage activity seems to be dependent on a specific Ago protein member, the degree of sequence complementarity between the guide miR and the target mRNA, and probably some additional, yet unknown factors.

Translational repression of mRNA

It has been observed in various studies that miRs induced a significantly smaller decrease in protein expression as compared to the corresponding mRNA levels, thereby implying the existence of translational repression.^{2,3,63,64} Translation of mRNA can in general be divided into three processes: ribosome initiation, elongation and termination. Inhibition may therefore occur at initiation steps or at post initiation steps.

Ribosome initiation may be inhibited by the miR/RISC complex. Polysome gradient analysis revealed that repression of mRNA by Let-7 miR or attached Argonaute proteins shift the mRNA target level to the same polysome levels as known translational inhibitors, indicating reduced ribosome loading on the repressed mRNA.⁴⁷ In addition, experiments performed in HeLa cells (immortal cancer cell line) indicated that the translation of m7G-capped mRNAs was repressed by miRs. However, mRNAs containing IRES (internal ribosome entry sequence) elements were not repressed by miRs, which indicates that miRs regulate cap-dependent initiation.^{64,65} These examples, amongst others, suggest miR-mediated repression occurs at the initiation step.⁶⁶⁻⁷⁰ Recent studies in *C. elegans* and mammals suggested that inhibition of translation occurs at post-initiation steps, as they demonstrated that polysomal distribution is not altered during repression by miR compared to non-repressed mRNA. Studies promoting translational repression at post initiation steps include co-translation peptide degradation (proteolysis), early termination by ribosomal drop-off and impaired elongation.⁷¹⁻⁷³ Cosedimentation of a cellular miRs or Ago proteins with polysomes has been reported in various studies, suggesting regulation at post-initiation steps.^{74,75} Repression of mRNA in *C. elegans* and unspliced mRNA in yeast seem to involve the stalling or slowing down of elongating ribosomes.^{76,77} In mammalian cells, short RNAs complexed with RISC are shown to bind with mismatches to 3' UTR of target mRNA and increase termination at a distant stop codon. This causes ribosomal drop-off at multiple sites within the open reading frame (ORF) more rapidly compared to non-inhibited mRNAs.⁷⁸ Unfortunately, the exact mechanism how miRs modulate elongation or termination processes is not clear yet.

mRNA deadenylation

Furthermore, RISC may contain regulatory proteins that might influence its inhibitory function, including fragile X mental retardation protein (FMRP). FMRP is a RNA-binding protein known as a translational modulator, particularly in neurons.^{25,47,79,80} In addition, GW182 (Gawky protein), which is a P-body component known to induce repression by interaction with RISC Ago proteins, recruits a deadenylation complex to the miR-mRNA duplex. The deadenylation complex, named CCR4-NOT, is shown to be capable of subsequent uncapping of target mRNAs.^{71,81}

In fact, P-bodies underlie a second way for miRs to target mRNAs for degradation, which is a slicer-independent mechanism. MiRs are shown to target mRNAs to P-bodies in the cytoplasm for decapping and degradation.⁶⁴ P-bodies contain degradation proteins that can remove the poly-A tail of mRNAs by de-adenylases. Translationally silenced mRNAs can also exit the P-bodies again for translational retry.⁸² Although P-bodies play a role in RNA silencing, they can also be formed as a consequence of gene silencing.⁷¹

However, translational repression by degradation can also occur GW182 independently in *Drosophila* and independent of a poly-A tail in mammalian cells. Therefore, it is highly likely that additional factors are involved in miR-mediated target repression.^{68,83}

Conclusively, miRs are thought to affect gene expression by mRNA cleavage, translational inhibition and/or by altering mRNA stability (summarized in figure 4). Results from studies conducted in different systems and different laboratories have often been contradictory. Whether translational inhibition occurs at the initiation or post-initiation step, or both, is still a matter of debate. However, it has been clearly observed that miRs do not mediate repression by itself, but rather

that multiple factors are involved that may contribute to translational repression.^{64,65} Most probably, repression of target mRNA occurs by a combination of the discussed repression pathways, and may show overlap in their mechanism of action. Elucidating the exact repression mechanism of miR will be important in understanding the role of miRs in human pathologies.

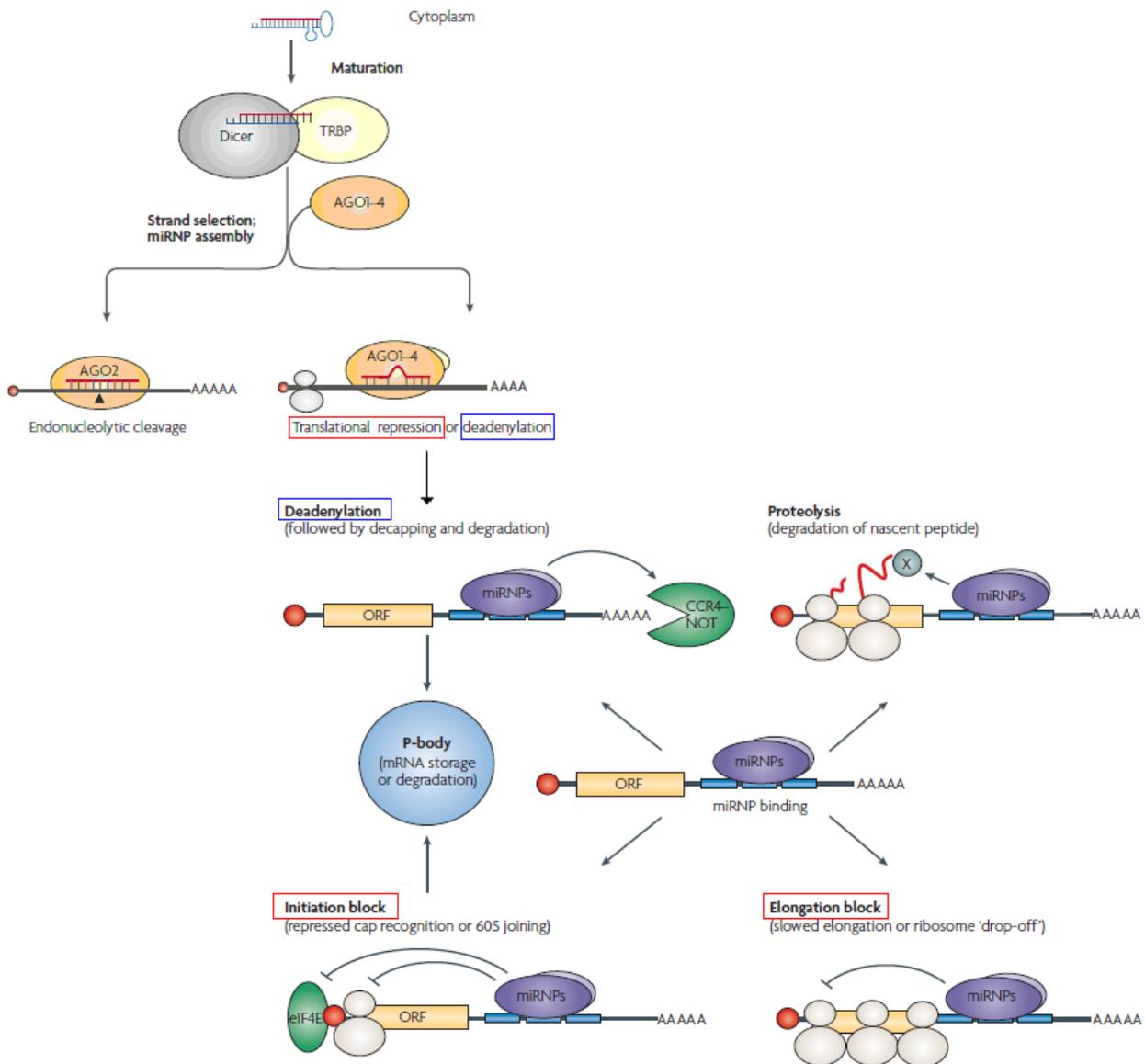


Figure 4. Translational repression of target mRNA by miRNAs. After miR maturation, the duplex is captured by Dicer and TAR RNA binding protein (TRBP), and cleaved into ~20 nucleotides RNA duplexes. The mature miR strand is assembled into micro-ribonucleoprotein complexes, or miRISCs. A key component of RISC is the Argonaute protein family. Ago1-4 mediate translational repression, but only Ago2 is capable of endonucleolytic cleavage in mammals. One method to repress the translation of target mRNA is deadenylation followed by decapping and degradation. P-body components such as GW182, that recruit deadenylation complex CCR4-NOT, interact with RISC AGO proteins to induce repression. Another method of translational repression is to prevent joining of ribosomal subunit 60S or repress cap recognition factor eIF4E. A third mechanism depicted is an elongation block by early ribosome drop-off. The last proposed mechanism is provided by proteasome mediated degradation of nascent peptides.⁸⁴
Recognition of target mRNA

Relevant in translational repression of the RISC/miR complex are the miR binding sites along a target mRNA. miR target sites used to regulate gene expression can be categorized into two broad classes. The first class are the "5' dominant sites", that base-pair well to the miR 5'. Within this class, two subtypes were recognized: 'canonical sites' pair well to miR 5' and 3'; 'seed sites' pair predominantly with 5', needing little or no pairing support from miR 3'. And a second class, "3' compensatory sites", that show weak base-pairing with miR 5' and strongly depends on compensatory pairing to the miR 3'. Evaluation of target mRNA pairing with the miR 3' suggested that seed sites are the most common miR target sites.^{63,85-87} Moreover, it was found that a match of only seven nucleotides with the miR 5' seed region was required for target recognition.⁸⁵ In particular, contiguous and perfect base pairing of nucleotides 2-8 at the 5' end of miRs are important for mRNA target recognition.^{63,88-91}

Furthermore, nucleotides 13-16 at the 3' end of the miR have to be complementary to the mRNA target.^{85,91} Although mismatches and bulges in the seed region affect repression, they are also required in the central region of the miR-mRNA duplex for Argonaute to be able to cleave target mRNA. Together, these features are required for miR-mRNA interactions.^{47,63} However other features are also required to boost target recognition. Grimson *et al.* described that these features include: an AU-rich nucleotide composition near the seed region, a location of the seed region near sites for coexpressed miRs and proximity of the miR binding sites along a target mRNA.⁹¹ These parameters can predict the RNA site performance, also in non-conserved sites and siRNA/miR off-targets (figure 5).⁹¹

Translational activation of mRNA

Interestingly, in some instances, miRs can also stabilize target mRNAs or even activate their translation. In proliferating cells, they repress translation, whereas in growth arrested cells (quiescence) they may mediate activation.⁹² It has been demonstrated that upregulation of tumor necrosis factor α (TNF α) mRNA was mediated by AU-rich elements (AREs) in the 3' UTR, which bind proteins Ago2 and FMRP to regulate mRNA stability or translation in response to quiescence-inducing stimuli.⁹² miR-369-3 bound to the 3'UTR of TNF α mediated this interaction and stimulated mRNA translation. Furthermore, target sites for miR let7-a and miRcxcr4 in the 3'UTR of a reporter containing AREs activated translation in response to growth arrest.⁹² Later research revealed that miR-10a and miR-122 also stimulate the translation of their target mRNAs in response to stress or nutrient shortage by interacting with the mRNA 5'UTR.^{93,94} Binding of these miRs to the 5'UTR of mRNAs does not seem to follow classical miR-mRNA interaction rules for seed region base-pairing, but the exact mechanisms by which these miRs operate is not clear.^{92,95}

miR target prediction and functional testing

Because miRs in animals are only ~25 nucleotides short, and sequences between miRs and their targets are imperfectly matched, a standard procedure as sequence comparison cannot be performed. In early experiments, with let-7 and lin-4, and also with miR-15a and miR-16-1, target

transcripts were determined by manually searching for matching locations which were confirmed through site-directed mutagenesis or other techniques.^{13,96} However, soon it became clear that miRs showed defined complement sequences to the mRNA 3'UTR target site, and that the longest contiguous alignment, the seed region, was 8 to 10 nucleotides long. As experimental validation of target sites by classical genetic techniques is laborious, time consuming and produce a substantial number of false positives, the need for computational techniques to predict target sites in silico increased.

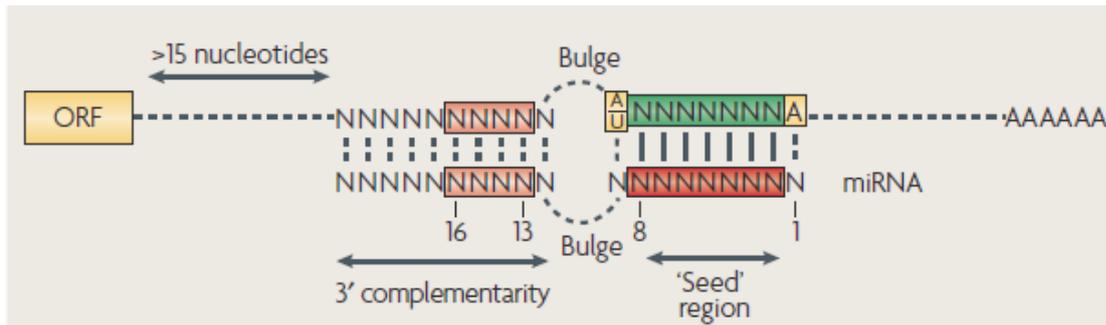


Figure 5. MiR-mRNA interactions. Requirements for miR-mRNA interactions include a perfect and contiguous base-pairing of nucleotides 2-8 at the 5'end of the miR, called the seed region. An A or U residue on position 9 and/or an A residue on position 1 of the target mRNA (shown in yellow) improve the miR-mRNA interaction efficiency, although base-pairing with miR nucleotides is not required. In the central region of the miR-mRNA interaction, bulges or mismatches must be present required for the Argonaute protein family to mediate endonucleolytic cleavage. Furthermore, at the 3'UTR of the miR, particularly to residues 13-16 (shown in orange) sufficient base-pairing is required for a stable binding.⁸⁴

The first computational methods were based on scoring sequence complementarity between mature miR and target sites in the 3'UTR mRNA database. In addition, free-energies of the miR-mRNA duplex and the search for homologous conserved miR genes or target site sequences in related genomes were important in these computational target gene predictions. Based on these scores, targets can be ranked. One of the first successful computational analysis for large-scale prediction of miR targets was done by Stark and colleagues with the use of sequence search tool HMMer (hidden Markov model) in *Drosophila melanogaster*.⁸⁷ This tool enabled G:U wobble matches and searched for 3'UTR mRNA target conservation in *Drosophila pseudoobscura* and *Anopheles gambiae*. Subsequently, thermodynamic stability was identified by using MFold algorithm.^{97,98} MFold performs RNA and DNA secondary structure prediction based on thermodynamic stability. Using this method, Stark *et al.* identified six new validated miR target sites which all showed perfect complementarity with residues 2-8 of the 5'UTR miR.⁸⁷

In later research, other computational target-prediction approaches were used to detect miR target sites. These methods include miRanda, TargetScan and PicTar, which all focus initially on finding complementarity, and then on thermodynamic stability. TargetScanS is an improved version of TargetScan but does not use a free-energy calculation. Furthermore, PicTar can predict target sites

that are co-regulated by diverse miRs. Other methods, which initially look at thermodynamic stability, include DIANA-microT, RNAHybrid, or whole genome motif-mining approaches (analysis of common motifs in 3'UTR sequences).⁹⁹ Finally, methods based on gene-expression include the support vector machine method. Target prediction techniques and their functions are summarized in table 1.

Name	URL
DIANA-microT	http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi
EIMMo	http://www.mirz.unibas.ch/EIMMo
miRanda	http://www.microrna.org
MirTarget2	http://mirdb.org
miTarget	http://cbiit.snu.ac.kr/~miTarget
PicTar	http://pictar.mdc-berlin.de
ma22	http://cbcsrv.watson.ibm.com/ma22.html
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid
TargetScan	http://genes.mit.edu/targetscan
TargetScanS	http://genes.mit.edu/targetscan

Name	Target species ^a	Algorithms	Performance	Distinguishing feature
DIANA-microT	Any	Thermodynamics	Precision: 66%	Target structure comes before seed complementarity
EIMMo	Humans, mice, fishes, flies, worms	Bayesian method	Sensitivity: 0.8; specificity: 0.95	Infers the phylogenetic distribution of functional target sites for each miRNA
miRanda	Flies, vertebrates	Complementarity	FPR: 24-39%(Fly)	Also provides the expression profile of miRNA in various tissues.
MirTarget2	Humans, mice, rats, dogs, chickens	SVM classifier	FPR: 22-31%; precision rate is 80% when the recall rate is below 20%	Microarray transcriptional profiling dataset is used for algorithm training
miTarget	Any	SVM classifier	An area under the ROC curve of 88.7% with the complete feature set	Training data is derived from validated miRNA targets from literature survey
PicTar	Vertebrates, flies, worms	Thermodynamics	FPR: 30%	Uses cross-species comparisons to filter out false positives
ma22	Any	Pattern recognition	FPR: 19-25.7% Sensitivity: 83%	Eliminates the use of cross-species conservation filtering, and leads to putative targets sites in 5' UTRs and ORF
RNAhybrid	Any	Thermodynamics, statistical model	SNR: 2.9:1 (vs 3.2:1); run-time: 13-181 times faster than RNAfold	An extension of the classical RNA secondary structure prediction algorithm
TargetScan	Vertebrates	Seed complementarity	FPR: 31% (human, mouse, rat), 22% (pufferfish, mammal)	Mainly searches for the presence of conserved 8- and 7-nt seed matches
TargetScanS	Vertebrates	Seed complementarity	FPR: 22% (mammal);	Requires 6-nt seed match and conserved Adenosine

Table 1. Computational methods to predict miR targets. Techniques used to predict miR targets within various organisms. Performance in terms of precision, sensitivity, specificity, false-positive rate (FPR) and signal-to-noise ratio (SNR), which measures unwanted perturbation to a wanted signal, are depicted. Their specific features are based on the different algorithms they use, including the Bayesian method. This is a method that specifies prior probability, which is then updated based on new relevant data. The SVM classifier (Support Vector Machine) creates a model that predicts whether a new example falls into one category or the other. a) Organisms for which the program is best suited.¹⁰⁰

To validate the potential target, which is the next step after prediction, the most used method is a standard reporter assay. In a reporter assay, predicted target sites are fused to a reporter construct, for example with luciferase or a fluorescent protein. The expression of the reporter construct is measured in absence and presence of miR.¹⁰¹ Other methods for validation include

northern blot analysis, quantitative real-time PCR, in situ hybridization and ribonuclease protection assays.¹⁰² Comparing protein levels in absence and presence of miRs can be accomplished by using western blots or immunocytochemistry. Although these methods are proven to be very useful in target validation, high-throughput experimental strategies are necessary to deal with the upcoming number of miR targets and their biological function.

To determine the function of the newly identified miR, *in vitro* or *in vivo* studies may provide information by knockdown or overexpression of the miR. Transfection of pre- pri- or mature miR sequence into a vector may induce miR sequence specific overexpression. Less common and a-specific inducers of overexpression are DNA methylating agents or histone deacetylase inhibitors. Knockdown or silence methods include blocking miRs by complementary binding of chemically modified oligonucleotides (e.g. morpholinos or antagomirs) to the mature miR strand, or using siRNAs.¹⁰³⁻¹⁰⁵ As individual miRs expressed from various genomic loci may repress multiple targets with a complementary seed sequence, it is not desirable to study functional miR families in genetic knockout models by blocking a single miR sequence (as in antisense oligonucleotides). To study the function of miR seed families, another *in vivo* method is needed. By applying multiple binding sites into the 3'UTR of a highly expressed miR target, this target might compete with the cognate miR(s). These modified competitive mRNAs are called decoy targets or sponges, and may be expressed transiently from transfected plasmids, viral vectors, or chromosomal insertions. By insertion of a bulge at a position that is normally cleaved by Argonaute protein 2, the decoy targets are able to interact with RISC containing the miR. They are proven to be effective in validation of target prediction and their activity is specific to miR seed families, and thus a good alternative for studying functional miR families *in vivo*.¹⁰⁶

High-throughput analyses, using solid phase, array platform or liquid phase bead-based hybridization revealed that miR expression was altered in many human cancer types. Altered miR expression may be tissue specific.¹⁰⁷ Moreover, up to 50% of the miRs appear to be located in regions with altered expression in human cancer.¹⁰⁸ Mapping of miRs to these altered chromosomal regions provided important clues. For example, the let-7 family map to deleted chromosomal regions in many tumor types.¹⁰⁹⁻¹¹¹ The main chromosomal alterations at miR sites are loss of heterozygosity (LOH) and amplification. Besides the miR location in cancer prone genomic regions, epigenetic regulation of miR expression and abnormalities in miR-processing genes and proteins may also contribute to altered miR expression.¹⁰⁸ Examples of epigenetic regulations are DNA methylation and histone-modification. Abnormalities in miR-processing genes and proteins include functional dysregulation of Dicer or RISC associated proteins.^{112,113}

MiR expression can be profiled based on cloning and sequencing of miRs from tissues or developmental states, or, if it are known miRs, by high-throughput microarray analysis. Besides the role of miRs in development, profiling of cancer specific miRs by comparing miR expression in certain cancer types to healthy tissue may be very useful. Microarray analysis is the most used high-throughput technique to detect cancer-specific miR expression, but can also be performed using bead-based flow cytometry, quantitative real-time PCR (to detect precursor or mature miRs), miRAGE (using small RNA tags for the detection of small RNAs) or RAKE assays (RNA primed,

array-based, Klenow enzyme platform, Klenow enzyme platform).¹¹⁴⁻¹¹⁶ By profiling miRs to their specific location, the histotype of tumors with unknown origin can be defined based on miR expression profile, or may be of prognostic value.^{109,117}

Since the identification of the first miRs in *C. elegans*, the list of reported miRs has now been expanded to 14,187 entries representing hairpin precursor miRs, expressing 15,632 mature miR products in 133 species. The number of sequences encoding human miRs is now 940, registered in the miRBase (<http://www.mirbase.org>). However, most miR targets are still unknown. In plants, miRs regulate a single target or one or two closely related families of targets by pairing to a single site within each mRNA.¹¹⁸ In humans, each miR sequence potentially targets approximately 200 genes.⁸⁹ miR sequence and target predictions are provided by various computational algorithms, with still show a poor specificity and sensitivity. With the growing number of miRs and their target sites, there is still a need for further improved large-scale analysis of miRs and their biological function. Moreover, experimental and computational strategies have to be integrated for more optimal detection of miRs and their multiple target sites.

Genomic location and expression

Approximately 97% of the human genome consists of noncoding DNA (i.e. introns or intergene regions), and numerous genes in these noncoding regions encode miRs. Introns can be located in protein-coding genes or in non-protein-coding genes. Almost half of all known mammalian miRs located within introns of either protein-coding or noncoding transcriptional units. In fact, ~40% of all identified mammalian miRs are found within introns of protein-coding genes, and ~10% are located within introns of non-protein-coding genes.^{119,120} In addition to intronic miRs, ~10% of all known mammalian miRs can be found in exons of noncoding regions.^{1,119,120} Occasionally, miR genes are located in either an exon or an intron, depending on their splicing pattern. The transcriptional origin of the remaining miRs were uncertain.¹¹⁹

Genes encoding miRs are situated in distinct transcriptional units or embedded in clusters of polycistronic host gene units, encoding several miRs. Clustered localization (within 50kb) and highly correlated expression patterns suggest that tandemly arranged miRs are processed from a single transcript to allow coordinate regulation. These intragenic miRs are, in contrast to intergenic miRs, co-expressed with the mRNAs of host genes, probably sharing the same promoter and expression control elements.¹²¹ Likewise, it has been shown that the majority of human, worm and fly miRs are situated within 10 kb of another miR.^{1,119,121}

In addition to above mentioned categorization of miR genes, it has also been reported that new miRs are sometimes generated from non-miR sequences that become miR genes by accumulated nucleotide substitutions.^{122,123} Others found that miRs may arise by duplication of existing miR genes or from transposable elements.¹²⁴⁻¹²⁶

MiR expression can be regulated by regulatory sequences in the promoter of miRs, for example by transcription factor binding motifs.¹²⁷ In addition, host genes harboring miR sequences in their intronic regions regulate the expression pattern of the corresponding miRs.^{4,119} For example, *let-7c* is an intergenic miRNA located in the antisense region of a gene intron, whose expression is regulated by the promoter of this gene.¹²⁸

In addition to this transcriptional regulation of miRs, post-transcriptional regulation of miR expression may also contribute to altered miR expression. Epigenetic regulation of miR expression may include mechanisms that directly influence DNA accessibility and gene expression in the context of chromatin, covalent and non-covalent modifications of DNA and histones. Or mechanisms that indirectly influence the transcription and translation from DNA to protein, including alternative splicing and poly-adenylation of mRNA transcripts, post-translational modification of proteins, and posttranscriptional regulation of transcribed RNA.¹²⁹ For example, zinc finger protein belonging to the PR domain-containing tumor suppressor protein family (PRDM5) was found to act as a sequence-specific DNA binding transcription factor. PRDM5 could recruit histone-modifying enzymes to its genetic targets, thereby functioning as a transcriptional repressor. Large-scale target identification revealed that PRDM5 can regulate many additional miR genes.¹³⁰ Abnormalities in miR-processing genes and proteins, including Drosha and Dicer, may also influence miR expression.¹⁰⁸ Together, these regulation mechanisms may have a major impact on miRNA expression during embryonic development and in cancer.

Evolutionary conservation

miRs have been well conserved throughout large distances in evolution.¹³¹ They have been identified in a broad range of animals, starting in worms (*C. elegans* and *C. briggsae*) by small RNA cloning and computational prediction. Combining these methods identified 90 miR genes, of which a majority possessed sequence conservation in other nematode miRs, vertebrates and humans.^{132,133} The *let-7* family shows the most extensive conservation across metazoans.^{16,131} Extensive research in the fruit fly (*Drosophila Melanogaster*) also identified highly conserved miRs in other insects and nematodes.^{88,134} However, some miRs are specific to organisms or specific between close related organisms, such as the *C. elegans* and *C. briggsae* shared *Isy-6*.¹³⁵

miR targets have also been tested for evolutionary conservation. For example, one of the *let-7* targets, *lin-41*, is broadly conserved in animals. *Lin-28*, another target of *let-7*, is broadly conserved in mammals and nematodes.^{16,20} The conservation of miRs may be dependent on how their targets have evolved throughout evolution, requiring co-evolution of the miR, the genes that control miR expression, and target genes. However, evolution may run fast in emergence and disappearance of miRs because they are very small and miRs do not need to maintain an open reading frame, since they do not encode proteins.¹³⁶

Role of miRs in apoptosis, cell cycle regulation and angiogenesis

As all 940 identified miRs are suggested to regulate approximately 30% of human genes, it seems very likely that they are involved in most biologic processes.¹ Reports have expanded functional roles of miRs to an array of distinct physiological processes, including development, proliferation, differentiation, cell signaling, cell fate determination, apoptosis, angiogenesis, host-viral interactions, and carcinogenesis.^{2,3,119,135,137-140} So far, it has been learned that miRs can function not only to maintain health, but that they are also associated with disease. In fact, multiple miRs and their target genes have been discovered by examining miR profiles in various types and stages of cancer. In this section, the roles of miRs in apoptosis, cell cycle regulation and angiogenesis will be discussed, as these processes are often deregulated in cancer.

Apoptosis

After the discovery of *lin-4* and *let-7*, genes were screened in flies that promote cell proliferation and suppress apoptosis during tissue growth. DIAP1 (Drosophila inhibitor of apoptosis protein 1) is the main regulator of cell death in Drosophila, inhibiting activated pro-apoptotic caspases.¹⁴¹ Important regulators of this pathway are *Reaper*, *Grim*, *Sickle* and *Hid*, which contain an IAP-binding motif that prevents DIAP1 function.¹⁴² Screening of apoptotic cell death inhibitors resulted in the identification of *Bantam*. *Bantam* showed sequence complementarity with the 3' UTR of mRNA encoding the pro-apoptotic gene *Hid*.¹⁴³ This was the first clue that miRs could prevent apoptosis in Drosophila.^{2,143}

Following the identification of apoptosis related miRs in Drosophila, mammalian cell lines were screened for miRs correlating to apoptosis. Currently, over 30 miRs are identified to be involved in apoptosis, and key genes playing a role in apoptotic pathways can be potential targets of these miRs.¹⁴⁴ MiRs may act on survival or death signaling pathways. Dependent on their target genes, miRs promote apoptosis or protect cells against it. However, a single miR can function both as pro-apoptotic and/or anti-apoptotic, which relies on the cellular context and the target gene. For example, miR-106b-25 and miR-17-92 may play roles in both the apoptotic and cell-proliferation pathways, dependent on their different target genes.¹⁴⁵ Many of the identified miRs involved in apoptosis and their target sites have been revealed by examining cancer derived cell lines.

Apoptosis may occur via several pathways, but the best characterized and most prominent are the intrinsic and extrinsic pathways (figure 6).¹⁴⁶ In the intrinsic pathway, pro-apoptotic stimuli target mitochondria directly or through transduction of proteins Bax (Bcl-2 associated protein X), Bak (Bcl-2 homologous antagonist/killer) and Bim (Bcl-like protein 11), which are pro-apoptotic members of the Bcl-2 family.¹⁴⁷⁻¹⁵⁰ When the intrinsic pathway is activated (e.g. by DNA damage, growth factor deprivation or oxidative stress), mitochondria release cytochrome c and other apoptotic factors, such as Smac (second mitochondria derived activator of caspases)/DIABLO (direct IAP binding protein with low pi).¹⁵¹ Smac/DIABLO can bind and inhibit IAPs (inhibitor of apoptosis proteins), which in turn inhibit caspases. Caspases are critical apoptotic proteins which can be subdivided into initiator (e.g. caspase-2, and -8 till -10) or effector caspases (e.g. caspase-3 till 7, and -11 till 13). Cytochrome c released from the mitochondria induces oligomerization of

Apaf-1 (apoptotic protease factor 1) that activates procaspase-9 to caspase-9, which then induces other effector caspases (figure 6).¹⁴⁷

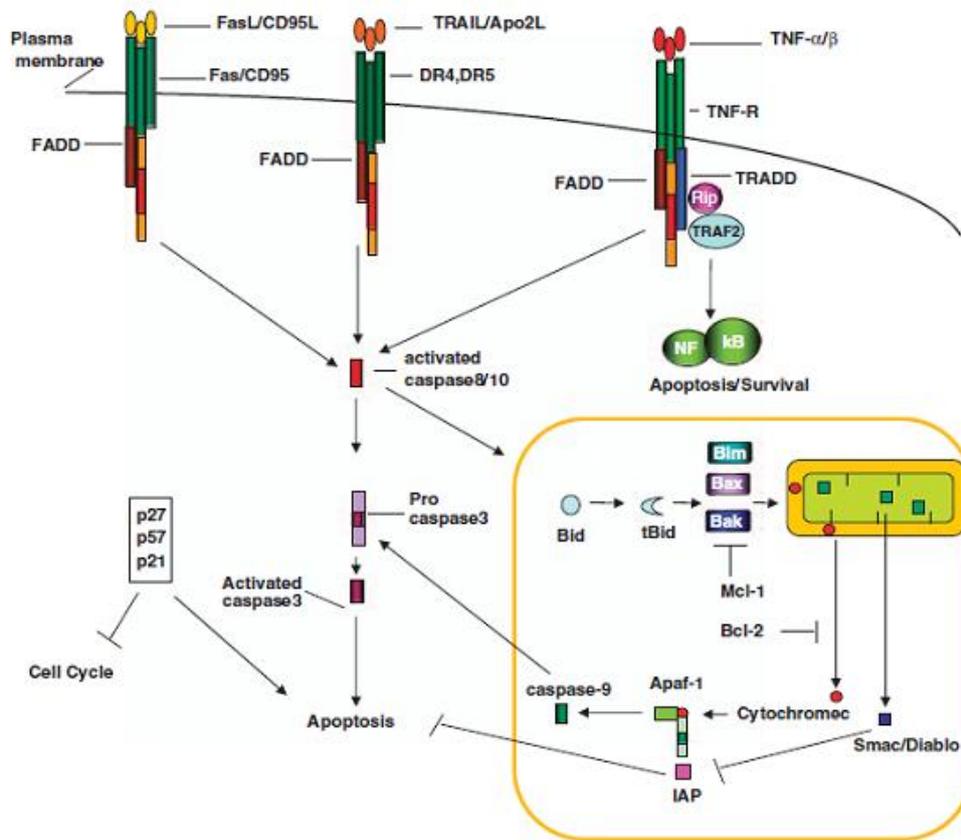


Figure 6. Intrinsic and extrinsic apoptotic pathways. The extrinsic pathway is activated when ligands, such as CD95/Fas, TRAIL, and TNF- α/β bind to their receptors on the cell membrane. Activated death receptors trimerize and recruit Fas-associated death domain (FADD). Activation of caspase 8 triggers the intrinsic pathway through cleavage of Bid, activation of Bax and the release of cytochrome C and SMAC/Diablo from the mitochondria. Cytochrome c binds to the adaptor proteins Apaf-1 and procaspase-9 to form an apoptosome. The apoptosome activates caspase-9 which activates caspase-3, resulting in apoptosis. Caspase 8 can also activate caspase-3 directly, via the extrinsic pathway. Bcl-2 protein and inhibitor of apoptosis proteins (IAPs) can respectively inhibit cytochrome c release and caspase activation. IAPs in their turn are inhibited by Smac/Diablo. Inhibitors of the cell cycle, such as p27, p57 and p21 can also induce apoptosis.¹⁴⁵

Several miRs are shown to play a role in the intrinsic apoptotic pathway. Cimmino *et al.* provided the first description of miRs being involved in apoptosis of human tissue and demonstrated that the expression of miR-15a and miR-16-1 were inversely correlated with *Bcl-2* expression in chronic lymphocytic leukemia (CLL).¹⁴⁰ *Bcl-2* protein is an anti-apoptotic family member that inhibits Bax. The 3'UTR of *Bcl-2* contains one potential binding site for both miR-15a and miR-16-1. By inhibiting *Bcl-2* expression, Bax will be continually active and induce the intrinsic pathway of apoptosis.^{140,152} For this reason, miR-15a and miR-16-1 are functioning as pro-apoptotic miRs.

Another pro-apoptotic miR in the intrinsic apoptotic pathway is miR-29. Mcl-1, an anti-apoptotic family member of the Bcl-2 family, can bind to Bcl-2 homology domains (BH3) containing proteins such as Bim, Bid and Bak.¹⁵³ Binding to these proteins protects the cell against the intrinsic apoptotic pathway. Mcl-1 protein was identified as a target of miR-29 in cholangiocarcinoma cell

lines and of miR-101 in hepatocellular carcinoma cell lines.^{154,155} Other identified targets of miR-29 include the DNA methylating genes *DNMT3A* and *DNMT3B*, which leads to abrogation of methylation mediated silencing of pro-apoptotic genes; and both p53 and CDC42, which leads to the activation of tumor suppressor p53.^{156,157} Thus, miR-29 regulates multiple apoptotic pathways. Furthermore, miR-122 is a pro-apoptotic miR in the mitochondrial apoptotic pathway. In addition to Mcl-1, another example of an anti-apoptotic family member of Bcl-2 is Bcl-w, also referred to as Bcl-2-like-2 (BCL2L2). Bcl-w is identified as a direct target of miR-122 in hepatocellular carcinoma cell lines, thereby inducing apoptosis.¹⁵⁸

In contrast to pro-apoptotic miRs, the muscle specific miR-133 acts as an anti-apoptotic miR in the intrinsic pathway by repressing caspase-9 gene (*CASP-9*) expression in cardiomyocytes.¹⁵⁹ The miR-17-92 cluster also functions as an anti-apoptotic miR cluster by targeting intrinsic apoptotic protein Bim in B-cell lymphoma subtypes.^{159,160}

The extrinsic pathway involves death receptors which are activated at the cellular membrane, such as the tumor necrosis factor receptor (TNFR) and death receptor Fas/CD95.¹⁶¹⁻¹⁶⁴ Induction of the extrinsic pathway via the activation of TNFR may activate the NF- κ B survival pathway or the c-Jun-N-terminal kinase (JNK) death pathway.¹⁶⁵ Via their ligands (TNF- α/β and FasL) and adaptor proteins (Fas-associated protein with death domain, FADD; TNF receptor-associated factor 2, TRAF2; and receptor-interacting protein, Rip), the extrinsic pathway eventually leads to activation of caspase-8 and -10. These caspases activate the more downstream pro-caspase 3 into caspase-3, which will induce apoptosis.^{166,167} The intrinsic and extrinsic pathways can crosstalk with each other via key proteins common in both pathways. For example, caspase-8/-10 can induce cleavage of Bid into an activated truncated t-Bid, activating the mitochondrial apoptotic pathway (figure 6).^{168,169}

Anti-apoptotic miRs involved in the extrinsic pathway include miR-125b, which is shown to target the 3'UTR of TNF- α . MiR-155 blocks the adaptor proteins FADD and Rip, as well as caspase 3.¹⁷⁰ Both results were obtained after LPS stimulation of macrophages in mice. Caspase-3 has been implicated as a target of multiple miRs. In an experiment performed in human breast carcinoma cells, apoptotic caspase-3 levels were decreased upon overexpression of miR-96, -145, -150 and -188, acting as anti-apoptotic miRs.¹⁷¹ In contrast, miR-10a, -28, -196a and -337 were shown to induce caspase-3 activity, acting as pro-apoptotic miRs.¹⁷¹ Both results were shown to be TRAIL-dependent, supporting their role in the extrinsic apoptotic pathway. MiRs involved in apoptotic pathways are summarized in table 2.

Cell cycle regulation

In addition to intrinsic and extrinsic apoptotic pathways, miRs may affect cell proliferation by controlling cell cycle regulators. One critical signaling pathway in the regulation of the cell cycle is the cyclin/CDK (cyclin dependent kinase) pathway. Extracellular signals may induce cyclin/CDK complexes, which then phosphorylate the retinoblastoma protein (pRb). pRb, in turn, dissociates from the E2F/pRb complex. Activated E2F contributes to the activation of pro-survival proteins, such as cyclins.

This pathway can be inhibited by several miRs. For example, miR-137, miR-449a/b and miR-124a inhibit CDK6 expression in different cancer cell lines, preventing cell cycle progression.¹⁷²⁻¹⁷⁵ miR-34a, which is transcriptionally activated by p53, can target both CDK6 and cyclin D1, preventing downstream pro-survival signaling of the cyclin/CDK pathway.¹⁷⁶⁻¹⁷⁸ Thus, these miRs are pro-apoptotic.

E2F is another target of miR-34a, as well as a target of the miR-17-92 cluster and the miR-106b-93-25 cluster.^{160,179-181} Although E2Fs are normally critical regulators of genes required for appropriate progression through the cell cycle, they can also be pro-apoptotic. E2F1 has been implicated with tumor suppressing activities, but the mechanism by which apoptosis is induced by E2Fs is still not understood.¹⁸² *Myc* can enhance the expression of *E2F*, while both *E2F* and *Myc* can activate the expression of the miR-17-92 cluster by binding to its promoter region, creating a negative feedback loop.^{180,181} Because high levels of E2F proteins, in particular E2F1, can induce apoptosis, this negative feedback loop may dampen E2F activity, lowering its pro-apoptotic signal, thereby promoting cell division rather than cell death.¹⁸²⁻¹⁸⁴ Similarly, the miR cluster miR-106b-25 can also be activated by E2F. Because E2F expression is downregulated by miR-106b and miR-93 in this cluster, a negative feedback loop is generated. The miR-106b-25 cluster can, in addition to the inhibition of E2F1, also negatively regulate pro-apoptotic genes *p21* and BCL2L11 protein expression, leading to cell cycle progression (figure 7).¹⁸⁵

The cyclin/CDK pathway is regulated by numerous genes and proteins, including members of the Cip/Kip family (p21, p27 and p57) and the INK4a/ARF family (p14 and p16).¹⁸⁶ These negative regulators are in turn regulated by several miRs, including miR-106b-25 and miR-17-92, targeting p21 in cancer cells as well as in normal lung fibroblasts; miR-221/222, targeting both p27 and p57 in various tumor cells; and miR-24, targeting p16 in human diploid fibroblasts and cervical carcinoma cells.^{160,187-190} By inhibiting negative regulators of proliferation, these miRs function as anti-apoptotic miRs (table 2).

Another cell cycle regulatory pathway is the PI3K/PTEN/Akt pathway. Upon activation of the receptor phosphoinositide 3-kinase (PI3K), the PI3K/AKT or Ras pathways enhance proliferation via multiple downstream targets, which eventually leads to activation of pro-survival transcription factors, including *Myc* (figure 7). The pro-apoptotic let-7 miR family targets transcription regulating factor high mobility group A2 (HMGA2), cell proliferation factors *Ras* and *Myc*, and other cell cycle progression genes.^{110,191-194}

The PI3K/Akt pathway is inhibited by a lipid phosphatase and tensin homolog (PTEN), functioning as an important tumor suppressor gene. MiR-21 targets *PTEN*, and additional tumor suppressor genes *TPM1*, *RECK* and *PDCD4*, acting as an anti-apoptotic miR (table 2).¹⁹⁵⁻¹⁹⁸

Apoptosis can also be induced by stress, for example by the tumor protein p53-inducible nuclear protein 1 (TP53INP1). Heat shock proteins (HSPs) may protect the cell from apoptosis. MiR-155 and miR-1 respectively target pro-apoptotic TP53INP1 and anti-apoptotic HSP60/HSP70.^{159,199}

p53 is targeted by miR-125b, and is therefore an important regulator of p53-mediated apoptosis during development and during the stress response (table 2).²⁰⁰

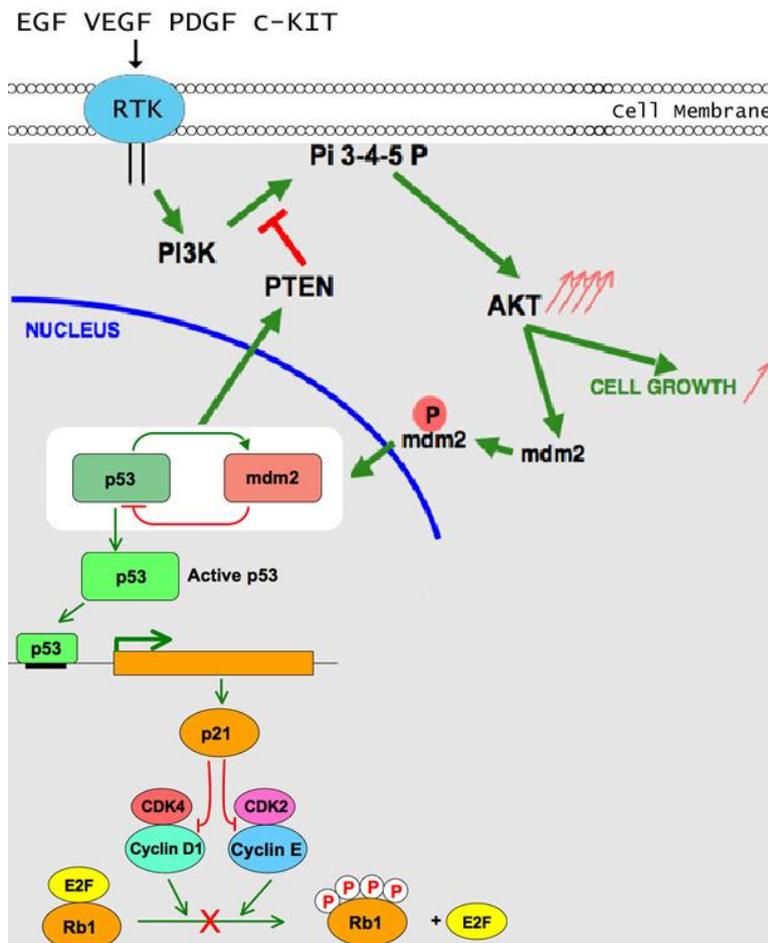


Figure 7. A simplification of how the PI3K, p53 and Rb pathways are connected. Activation of the receptor tyrosine kinase (RTK) by external growth factors leads to dimerization of the receptor and activation of phosphoinositide 3-kinase (PI3K). PI3K activates multiple targets, including AKT. AKT can inhibit pro-apoptotic proteins, such as Bim, contributing to cell survival, but is also involved in the cell cycle via activation of mdm2. Mdm2 is a negative regulator of tumor suppressor p53. Upon DNA damage, hypoxia or cell cycle abnormalities, p53 can bind DNA and activate the transcription of pro-apoptotic genes, including genes encoding p21. Cell cycle progression requires an activated CDK-cyclin complex, which can phosphorylate Rb1 to release transcription factor E2F. p21 prevents the activation of CDK/cyclin complexes, preventing cell cycle progression.

In conclusion, miRs are involved in both intrinsic and extrinsic apoptotic pathways, as well as in various cell cycle control pathways, which show a complex interplay between each other (figure 7). By interfering with different apoptotic targets, they can broadly be classified as pro-apoptotic or anti-apoptotic miRs. Currently identified miRs involved in the apoptotic or cell cycle regulatory pathways in mammals are summarized in table 2. Taken into account that some individual miRs have many identified targets, it is likely that the miRs listed here are single components of much larger regulatory pathways. Moreover, miRs might also be subjected to cell cycle dependent

regulation by themselves. Therefore, it will be necessary to generate a more complete picture about miRs involved in apoptosis and cell proliferation in future.

Table 2. MiRs involved in apoptotic and cell cycle progression

microRNA	Effect on apoptosis	Targets	Investigated in	References
miR-17-5p	anti-apoptotic	E2F1, CyclinD1	Breast cancer cell line	329; 330
miR-27a	anti-apoptotic	ZBTB10, RINZF, RYBP, DEDAF	Breast cancer cell line	112
miR-133	anti-apoptotic	CASP-9	Cardiomyocytes	159
miR-221/222	anti-apoptotic	p27 (1), p57 (2), c-KIT (3), Era α (4)	Breast cancer cell lines (4), human hepatocellular carcinoma (1,2), vascular smooth muscle cells (3)	158, 227, 254, 190
miR-106b-25	anti-apoptotic	p21/CIP1 (1), BIM (2), E2F1 (3)	HeLa cells, B-lymphocyte cell line (2,3), human mammary epithelial cells, lung carcinoma cells, lung fibroblasts (1)	185, 188, 189
miR-17-92	anti-apoptotic	p21/CIP1, BIM, E2F1	B-cell lymphomas	180
miR-155	anti-apoptotic	TP53INP1 (1), FADD (2), Rip (3), Caspase 3 (4)	Mice macrophages treated with LPS (2,3,4), pancreatic ductal adenocarcinoma (1)	159, 170, 199
miR-125b	anti-apoptotic	TNF- α (1), p53 (2)	Mice macrophages treated with LPS (1), human neuroblastoma cells and human lung fibroblasts (2)	170, 200, 327
miR-24	anti-apoptotic	p16 ^{INK4a}	Human diploid fibroblasts, cervical carcinoma cells	187
miR-21	anti-apoptotic	HNRPK (1), Tap63 (2) PDCD4 (3), RECK (4), PTEN (5), TPM1 (6)	Human hepatocellular carcinoma (3,4,5), glioblastoma (1,2), breast cancer cells (6)	195, 196, 197, 198

	pro-apoptotic	Bcl-2	Breast tumor tissues	250
miR-34a	pro-apoptotic	E2F3, E2F5, SIRT1, MET, CDK6, CyclinD1	Human colon adenocarcinoma, embryonic stem cells, lung adenocarcinoma cell line	176, 177, 179
miR-15a, miR-16-1	pro-apoptotic	Bcl-2	B-cell Chronic lymphocytic leukemia	140
Let-7	pro-apoptotic	Ras (1), NF2 (2), HMGA2 (3), MYC (4)	Lung cancer (1,3), breast cancer cells (1,3), Burkitt lymphoma cells (4), human cholangiocarcinoma cells (2), ovarian cancer (3), HeLa cells and mouse embryo fibroblasts (3)	110,191, 192, 193, 194
miR-122	pro-apoptotic	Bcl-w, cyclin G1	Hepatocellular carcinoma cell lines	158, 260
miR-101	pro-apoptotic	Mcl-1	Hepatocellular carcinoma cell lines	155
miR-29a,b,c	pro-apoptotic	Mcl-1 (1), DNMT3A/3B (2), p85, CDC42 (3)	Cholangiocarcinoma cell lines (1), lung cancer cells (2), HeLa cells (3)	154, 156, 157
miR-1	pro-apoptotic	HSP60/HSP70	Cardiomyocytes	159
miR-137 (1), miR-449a/b (2), miR-124 (3)	pro-apoptotic	CDK6	Glioblastoma cell lines (1,3), medulloblastoma (3), sarcoma osteogenic cell line (2)	172-175
miR-145	pro-apoptotic	TP53 (1), Era (2), RTKN (3), IRS-1 (4), -c-Myc (5)	Human breast cancer cell lines (1,2,3,5), colon cancer cells (4,5)	258, 385, 386
miR-143	pro-apoptotic	ERK5, KRAS	Human T-cell leukemia Jurkat cells, colorectal tumor cells	386, 387

Angiogenesis

Angiogenesis is an important process in normal embryonic development and for physiological processes, such as organ growth, wound healing, menstrual cycles or transplantations.²⁰¹ Angiogenesis is downregulated during adult life (except for the female reproductive system) and induced by micro-environmental factors, such as hypoxia or inflammation. Vascular endothelial cells in health respond to a balance between pro- and anti-angiogenic factors produced and secreted by various cells to maintain blood vessel homeostasis.²⁰² Under normal conditions, the balance is mediated by homeobox (*HOX*) genes, which can be subdivided into pro-angiogenic and anti-angiogenic *HOX* genes.²⁰³ Pro-angiogenic *HOX* genes regulate the expression and secretion of pro-angiogenic factors, which include vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGFs).²⁰⁴ VEGFs and FGFs exert their functions by binding to specific cell surface expressed receptors with tyrosine kinase activity. These receptor kinases allow interactions with downstream signal transduction pathways, such as the mitogen-activated protein kinase (MAPK) and PI3K pathways.²⁰⁵ The PI3K and MAPK pathways regulate various processes, including proliferation, migration and differentiation of endothelial cells.^{206,207} Multiple reports have implicated that miRs control the angiogenic response of endothelial cells. Endothelial miRs that promote angiogenesis include let-7f, miR-27b, miR-126, miR-130a, miR-210, and miR-296.²⁰⁸⁻²¹¹ No target sites have been identified for the pro-angiogenic miRs let-7f and miR-27b.²⁰⁸ Some miRs, such as the miR-17-92 cluster, can function both as pro- and anti-angiogenic, dependent on the cellular context.

Angiogenesis can be promoted by antagonizing anti-angiogenic genes or proteins. For example, inhibiting anti-angiogenic *HOX* genes, such as *GAX* (growth-arrest specific homeobox), will promote angiogenesis. *GAX* is an inhibitor of both NF- κ B signaling and angiogenesis, *in vitro* and *in vivo*.^{212,213} Another anti-angiogenic gene is *HOXA5*, which acts through targeting different downstream pro-angiogenic targets, such as VEGFR2.²¹⁴ miR-130a targets both *HOXA5* and *GAX*, abrogating their angiogenic inhibition.²¹⁵

VEGF, an important central mediator of angiogenesis, has been identified as an inducer of several miRs involved in angiogenesis.²¹¹ VEGF acts through its receptor (VEGFR). As an intact VEGFR/PI3K/MAPK signaling cascade is important for new blood vessel formation, angiogenesis can be inhibited by VEGF receptor degradation. The degradation of VEGF receptors is mediated by hepatocyte growth factor-regulated tyrosine kinase substrate (HGS). MiR-296 inhibits HGS, stimulating growth-factor mediated angiogenesis.²¹⁶

Further downstream, the angiogenesis promoting MAPK and PI3K pathways can respectively be inhibited by Sprouty related protein 1 (Sprd 1) and phosphoinositide 3-kinase regulatory subunit 2 (PIK3R2). Evidence implicate that miR-126 is required for the maintenance of vascular *structure in vivo*, as has been examined in zebrafish and mice model systems.^{209,210,217} miR-126 inhibits Sprd1 and PIK3R2, thereby stimulating angiogenesis.^{210,218} Additional evidence showed that miR-126 also plays a role in controlling immune infections through repression of vascular cell adhesion molecule (VCAM)-1 in TNF- α -treated endothelial cells, and in normal hematopoiesis through targeting *HOXA9*.^{218,219}

In addition to growth factors and *HOX* genes, angiogenesis can be stimulated by hypoxia. Among all identified hypoxia regulated miRs in endothelial cells, miR-210 is induced by hypoxia in all cell types tested.²²⁰⁻²²³ A relevant target of miR-210 is receptor tyrosine kinase Ephrin A3. As Ephrin A3 normally prevents capillary tube formation and endothelial cell migration, miR-210 is an important regulator of angiogenesis and endothelial cell survival in response to hypoxia (figure 8).²²³

Endothelial miRs that inhibit angiogenesis include miR-221, miR-222 and miR-34a.^{224,225} In a model of human umbilical vein endothelial cells (HUVECs), which is a valuable model of *in vitro* angiogenesis, miR-221 and miR-222 inhibited angiogenesis by targeting c-Kit.²²⁴ C-Kit is a tyrosine kinase receptor for stem cell factor (SCF), which promotes survival, migration and capillary tube formation in HUVECs.^{224,226} By decreasing the abundance of c-Kit, miR-221 and miR-222 prevent the formation of early erythroblasts and maintain hematopoietic stem cells (figure 6).^{224,227} An important modulator of vascular endothelial cell homeostasis is Silent information regulator 1 (Sirt1).²²⁸ Sirt1 is recently shown to promote proliferation and prevent senescence in primary porcine aortic endothelial cells.²²⁹ miR-34a suppresses Sirt1, inducing senescence and inhibiting endothelial progenitor cell (EPC) mediated angiogenesis.²²⁵

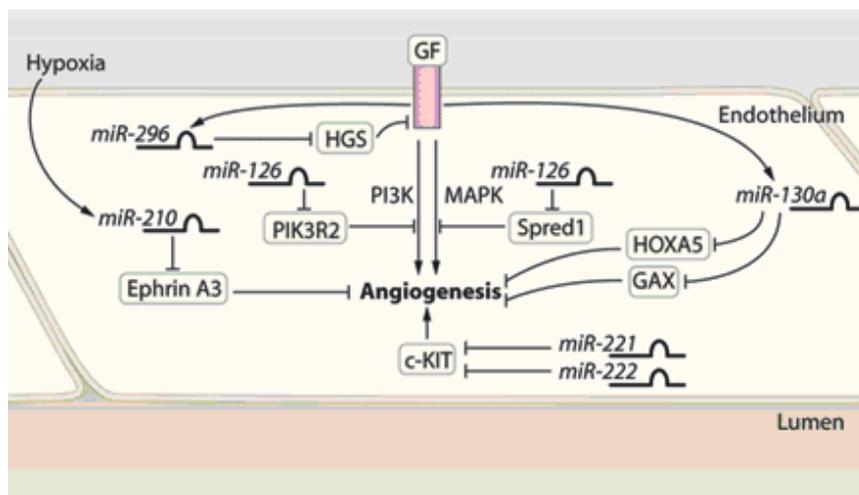


Figure 8. MiRs involved in angiogenesis. Growth factors (e.g. vascular endothelial growth factors VEGFs and fibroblast growth factors FGFs), induce angiogenesis via multiple signal transduction pathways, including the PI3K and MAPK pathways. MiR-296, miR-30a, miR126 and miR-210 are pro-angiogenic. MiR-221 and miR-222 are anti-angiogenic. Target genes depicted are HGS (hepatocyte growth factor-regulated tyrosine kinase substrate), PIK3R2 (Phosphoinositide 3-kinase regulatory subunit 2), Spred1 (Sprouty related protein 1), *HOXA5* (Homeobox A5), *GAX* (Growth arrest specific homeobox), tyrosine kinase Ephrin A3 and tyrosine kinase receptor c-KIT.²⁰⁵

Above mentioned pro- and anti-angiogenic miRs are autonomously regulated by endothelial cells. However, viral proteins, tumor cells and diabetic endothelial cells may also promote miR expression in a non-autonomous fashion. For example, in the absence of Dicer, VEGF-induced proliferation and morphogenesis were mediated, in part, by miR-17-92 activation.²³⁰ Within tumor cells and in endothelial cells lacking dicer, the miR-17-92 cluster has been shown to promote angiogenesis through repressing anti-angiogenic protein thrombospondin 1 (Tsp1) and connective tissue growth

factor (CATG).^{184,230-232} However, the miR-17-92 cluster has also been shown to exhibit cell-autonomous anti-angiogenic activity through targeting Janus kinase 1 (Jak1) and integrin $\alpha 5$ in endothelial cells.^{233,234} Thus, the opposing effects of this miR cluster seem to be dependent on the cellular compartment.

Despite current knowledge about the role of miRs in angiogenesis, the regulatory pathways of angiogenesis controlled by miRs have yet to be fully elucidated. All identified miRs involved in normal angiogenesis thus far are summarized in table 3. MiRs involved in tumor angiogenesis are also included, but will be discussed in the following chapter.

Table 3. MiRs involved in angiogenesis.

microRNA	Effect on angiogenesis	Function	Targets	References
miR-130a	pro-angiogenic	Antagonizes the anti-angiogenic activity of HOXA5 and GAX	HOXA5, GAX	215
miR-296	pro-angiogenic	Inhibits the degradation of VEGF receptors in <i>in vitro</i> matrigel and scratch wound assays	HGS	216
miR-126	pro-angiogenic	Required for the maintenance of vascular structure <i>in vivo</i> in zebrafish and mice models	Spred1, PIK3R2, p85	209, 210, 217, 265
miR-210	pro-angiogenic	Regulator of angiogenesis and endothelial cell survival in response to hypoxia <i>in vitro</i>	Ephrin-A3	223
miR-27b and Let-7f	pro-angiogenic	Required for angiogenesis in endothelial cells <i>in vitro</i>	Tsp1?	208
miR-378	pro-angiogenic	Promotes angiogenesis in a colon cancer model <i>in vitro</i>	SuFu, TUSC2	269
miR-17-92	pro-angiogenic	Promotes angiogenesis within tumor cells and in endothelial cells lacking dicer	Tsp1, CATG	231, 232
	anti-angiogenic	Anti-angiogenic activity in normal endothelial cells	Jak1, Integrin- $\alpha 5$	233, 234
miR-221/222	anti-angiogenic	Prevent the formation of early erythroblasts and maintain hematopoietic stem cells	c-KIT	224, 227
miR-34a	anti-angiogenic	Inhibits endothelial progenitor cell (EPC) mediated angiogenesis	Sirt1	225

Altered microRNA expression and cancer

Identification of altered miR expression in cancer

In tumorigenesis, physiological processes as apoptosis, cell proliferation and angiogenesis, are often deregulated. Not surprisingly, miRs controlling these processes have been associated with initiation and progression of human cancer.

The first evidence that alterations in miR function or gene expression were involved in cancer came from the research group of Calin *et al.* In patients with B-cell chronic lymphocytic leukemia (CLL), but also in other tumor types, chromosomal region 13q14 is often lost. This region contains the Retinoblastoma gene (RB1), which is an inhibitor of cell cycle progression, as well as miR-15a and miR-16-1.^{235,236} Calin *et al.* found that 68% of the patients with CLL had deletions or showed downregulation of miR-15a and miR-16-1, which raised the question whether they might function as tumor suppressors.¹⁵² Indeed, Cimmino *et al.* showed that miR-15a and miR-16-1 negatively regulate the expression of anti-apoptotic Bcl-2 as the loss of these miRs resulted in overexpression of Bcl-2 in leukaemia cells and overexpression of these miRs resulted in decreased Bcl-2 endogenous protein.¹⁴⁰ miR-15 and miR-16 are, in addition to CLL, also deleted or downregulated in DLBCL (Diffuse Large B-cell Lymphoma), multiple myeloma, pituitary adenoma, prostate and pancreatic cancer.²³⁷ In a number of human cancers in which overexpression of Bcl-2 may evade apoptosis, including Hodgkin's lymphoma and DLBCL, deregulation of miR-15 and miR-16 may be an important event in tumorigenesis.²³⁸⁻²⁴⁰

Since the discovery of miR-16-1 and miR-15a involvement in CLL, various other studies have shown strong correlations between enhanced or diminished miR expression and cancer. These observations have led to mapping chromosomal regions of all known miRs, which revealed that miRs are often located in chromosomal regions that are deleted or amplified in many types of human cancers.¹⁰⁸ In fact, about half of the identified miR genes are located in or close to cancer-associated regions.¹⁰⁸ Based on earlier provided information, this paragraph summarizes altered miR expression associated with apoptosis, cell proliferation, and angiogenesis in cancer.

Altered miR expression in apoptosis and cell cycle regulation

It is known now that miRs play important roles in regulating apoptosis and cell proliferation in cancer. Important tumor suppressor genes involved in apoptosis in cancer are, for example, *Rb1*, *TP53* and *PTEN*. The first identified tumor suppressor gene encodes the Rb1 protein, an inhibitor of E2F. *TP53* encodes p53, a transcription factor of many pro-apoptotic genes. The PTEN protein removes phosphate groups which prevent cell signaling of several downstream pathways, including the PI3K/Akt pathway (figure 7). In cancer, these tumor suppressor genes may be deleted or downregulated, which may result in constitutive cell-cycle progression. *Rb1*, for example, is mutated in about 40% of all retinoblastoma patients and in many other cancer types.^{241,242} More

than half of the human tumors contain a *TP53* mutation or deletion and also *PTEN* alteration have been implicated in a variety of human cancers, including glioblastoma, melanoma, endometrial, breast, thyroid and prostate cancer.²⁴³

Tumor suppressor genes can be regulated by miRs, as discussed in the previous paragraph. By inhibiting tumor suppressors, miRs may contribute to the survival of cancer cells, exerting an oncogenic role. Some oncogenic miRs are shown to be upregulated in certain types of cancer. For example, targeting tumor suppressor p53 may contribute to oncogenic functioning of miRs. MiR-125b targets p53 directly and miR-155 targets TP53INP1.^{199,200} Both are upregulated in certain types of tumor (table 4).^{170,199}

E2F transcription factors, which can induce both cell proliferation and apoptosis, is a target of miR-330, the miR-17-92 cluster, the miR-106b-93-25 cluster and miR-34.^{160,177,178,180,244} Members of the miR-17-92 and miR-106b-93-25 cluster are overexpressed in colon cancer, multiple myeloma and other tumor types (table 4).^{244,245} The miR-17-92 cluster targets, in addition to E2F1, apoptotic factors PTEN and Bim, and anti-angiogenic factors TSP1 and CATG. As a result, overexpression of miR-17-92 enhanced cell growth in a chronic myeloid leukemia and a lung cancer cell line.²⁴⁶⁻²⁴⁸ Overexpression of miR-106b-93-25, which also targets E2F1 and Bim, impaired the TGF β tumor suppressor pathway in gastric cancer.¹⁸⁵

In addition to these oncogenic miRs, the expression of miR-21 is also increased in many tumor types, including breast cancer, colorectal cancer, glioblastoma, cervical cancer and pancreatic cancer.²⁴⁹⁻²⁵³ MiR-21 targets important components of the p53 and mitochondrial apoptotic pathways (table 2 and 4). By targeting these tumor suppressors, upregulation of miR-21 in cancer induces cell growth in various tumor types.^{250,251,254} Supporting its oncogenic role, tumor miR-21 knockdown induces apoptosis in glioblastoma.^{253,255}

Thus, in cancer, miRs targeting tumor suppressor genes may be upregulated, contributing to the survival of cancer cells. Oncogenic miRs involved in human cancer are summarized in table 4.

In addition to the inhibition of tumor suppressors, some miRs may also inhibit oncogenes, exerting a tumor suppressive role. Important oncogenes involved in tumor cell survival, include *Ras* and *Myc*. *Ras* encodes a protein downstream of the receptor tyrosine kinase pathway, which may activate pro-survival transcription factors, including *Myc*. In cancer, these oncogenes are often upregulated, which may result in constitutive cell proliferation. In fact, *Ras* is active in 20-30% of human tumors, and is thereby one of the most commonly mutated oncogenes.²⁵⁶

Some tumor suppressive miRs are downregulated in certain tumor types.^{244,257} For example, *Myc* and *Ras* are targeted by let-7 and miR-143/145, repressing cell proliferation and exerting a tumor suppressive role.^{110,194,258} miR-143/145 is downregulated in B cell malignancies, colon adenoma/carcinoma and breast, lung and cervical cancers.^{237,252,259} Let-7 is downregulated in different cancer types, including lung, breast and prostate cancers.^{109,237,249,260} However, the Let-7a family member is upregulated in certain cancer types, which contributes to insensitivity to chemotherapy (table 4).²⁶¹

MiR-34a is another example of a tumor suppressive miR, which downregulated in different cancer types (table 4). Introduction of miR-34a in two colon cancer cell lines was associated with inhibition of cell growth, G1 accumulation and senescence in these cells.¹⁷⁹ In addition, decreased miR-34a expression has also been associated with p53 inactivation in CLL.^{177,178} Recently, it has been shown that overexpression of miR-34a induces apoptosis in malignant peripheral nerve sheath tumor cell lines, supporting the tumor suppressive role of miR-34a.²⁶²

Altered miR expression in angiogenesis

Altered miR expression can also be measured in endothelial cells in response to angiogenic factors produced by cancer cells. For example, the level of pro-angiogenic miR-296, which inhibits degradation of the VEGF receptor, is increased in endothelial cells cocultured with glioma cells or in response to VEGF stimulation. When miR-296 is inhibited in *in vivo* experiments, vascularization of tumor xenografts decreases.²⁶³ Thus, angiogenic miRs may be upregulated in cancer cells, providing them a growth advantage.

However, the expression of angiogenic miRs can also be decreased in cancer cells. MiR-126, for example, was shown to be downregulated in many cancer cell lines, including colon, lung and breast cancer.²⁶⁴⁻²⁶⁶ MiR-126 enhances the PI3K pathway by targeting its negative regulators Spred1 and PIK3R2 in endothelial cells.²⁰⁹ Knock-down studies in mice and zebrafish resulted in angiogenic deficits and embryonic lethality, implicating its importance in vascular integrity and angiogenesis.^{210,267} In cancer cells, miR-126 was additionally shown to target p85 regulatory subunit, which propagates and stabilizes the PI3K signal. In a panel of matched normal colon and primary colon tumors, miR-126 downregulation was associated with an increase in the p85 protein level.²⁶⁵ An increase of the PI3K signaling protein p85 would contribute to a selective growth advantage during tumorigenesis.

Angiogenesis can be controlled by miRs directly, or through the regulation of angiogenic factors, such as anti-angiogenic secreted factors TSP1 and CATG.²⁶⁸ These factors, targeted by the miR-17-92 cluster, stimulate angiogenesis. In agreement, miR-17-92 overexpression in tumor cells promotes tumor angiogenesis.²⁴⁸ In a non-paracrine mechanism, miR-378 promotes angiogenesis in a colon cancer model by targeting tumor suppressors SuFu (suppressor of fused homolog) and TUSC2 (tumor suppressor candidate 2).²⁶⁹

Thus, miRs may contribute via autocrine or paracrine pathways to angiogenesis in cancer cells. As angiogenesis is an important feature of cancer cell proliferation, survival and metastasis, it is very important to gain more insights into miRs involved in this process.

In conclusion, miRs involved in physiological processes important in tumorigenesis, such as apoptosis, cell signaling and angiogenesis, may be up- or downregulated in certain tumor types. Tumor suppressor miRs that under normal circumstances enhance apoptosis, inhibit proliferation, or inhibit angiogenesis, might be downregulated or deleted in tumors. Vice versa, oncogenic miRs that under normal circumstances inhibit apoptosis, enhance proliferation, or enhance angiogenesis,

may be overexpressed in cancer cells. By looking at tumor suppressor or oncogenic miRs in cancer, a diagnostic and prognostic miR expression profile can be created that correlates to a patient survival.¹⁴⁵ However, as miRs may have dozens, if not hundreds of other target genes, care should be taken with calling miRs oncogenes or tumor suppressors. miRs involved in human cancer are summarized in table 4.

Table 4. MiRs involved in human cancer.

miR	Upregulated or downregulated	In cancer type	Targets	Molecular Function	References
Let-7 family	Downregulated	Lung, breast, gastric, ovary, prostate and colon cancer; CLL, melanoma and leiomyoma	CCND1, CDC25a, CDC34, CDK6, CD-BP, Dicer, HMGA2, HOXA9, IMP-1, ITGB3, MYC, Ras, TLR4	Repress cell proliferation and growth. Let-7f promotes angiogenesis	110, 191, 192, 193, 194
	Upregulated	Colon, pancreatic cancer; hepatocellular carcinoma, AML and uterine leiomyoma	Caspase-3 (for let-7a)	Let-7a represses NF2 and decreases chemotherapy-induced apoptosis <i>in vitro</i>	261, 388, 389, 390
miR-10b	Upregulated	Metastatic breast cancer, pancreatic adenocarcinoma and glioblastoma	HOXD10	Activates cell migration and extracellular matrix remodeling	266, 345
miR-15a/16-1	Downregulated	CLL, DLBCL, multiple myeloma, pituitary adenoma, prostate and pancreatic cancer	Bcl-2, CARD10, CCND1, CDK6, CDC27, CGI-38, CYCE, DMTF1, HMGA2, MCL1, MYB, NGN2, VEGF, WNT3A	Induce apoptosis in leukemia cells. miR-16 regulates cell cycle by downregulating G0/G1 proteins	140, 152, 385
	Upregulated	Nasopharyngeal carcinoma	BRCA1		391
miR-17/18a/19a / 19b-1/20a/ 17-92 cluster	Upregulated	Lymphoma, multiple myeloma, medulloblastoma, lung and colon cancer	AIB1, AML1, Bim1, CTGF, CDKN1A, E2F1, E2F2, E2F3, HIF-1A, PTEN, TGFBR2, TSP1, Rb2/P130	Increase tumor growth and vascularization; miR-20a is antiapoptotic	160, 181, 246-248,
	Downregulated	LOH at miR-17-92 locus in melanoma, ovarian and breast cancer	AIB1, CYCD1	miR-17-92 reduces breast cancer proliferation	329

miR-106b cluster	Upregulated	Neuroblastoma, multiple myeloma, prostate, colon and gastric cancer	CDKN1A, E2F1, Bim	Reduces apoptotic response after TGF- β stimulation via Bim	185, 188
miR-21	Upregulated	Glioblastoma, uterine leiomyosarcoma, DLBCL, head, neck, breast, lung, prostate, colon, stomach, esophageal, and cervical cancer	BCL2, MASPIN, PDCD4, PTEN, TPM1, RECK, RASA1	miR-21 knockdown induces apoptosis in glioblastoma. miR-21 induces invasion, metastasis in colorectal cancer	249, 250, 251, 253, 254, 259
miR-29	Downregulated	CLL, cholangiocarcinoma, colon, breast, and lung cancer	CDC42, DNMT3A/B, MCL1, PIK3R1, TCL1	Induce aberrant methylation in lung cancer via DNMT3A/B; induce apoptosis via p53 and MCL1	154, 156
	Upregulated	Breast cancer	TTP metalloproteinases	Induces EMT transition and metastasis	392
miR-34	Downregulated	Downregulated in pancreatic cancer and Burkitt's lymphoma without MYC translocation. Hypermethylation of miR-34b/c in colon cancer	BCL2, CCND1, CCNE2, CDK4/6, MYC, DLL1, E23, Notch1, MYCN, MET, HMG2A, SIRT1	miR-34a induces upregulation of p53, downregulation of E2F in colon cancer	176, 177, 179, 245, 332
miR-101	Downregulated	Hepatocellular carcinoma, bladder and prostate cancer	COX2, EZH2, MCL1	Alterations in global chromatin structure via repression of EZH2	393, 394
miR-122a	Downregulated	Hepatocellular carcinoma	CAT-1, CCNG1	miR-122a can modulate cyclin G1 expression in HCC-derived cell lines and contributes to the cellular stress response via inhibition of CAT-1	69, 260, 395
miR-124a	Downregulated	Hypermethylation in leukemia, lymphoma, colon, breast, gastric and lung cancer	CDK6, ITGB1, FOXA2, LAMC1, MTPN, MAPK14	Induce differentiation of human glioblastoma multiforme-derived stem cells and induce glioblastoma multiforme cell cycle arrest	175, 396
miR-125a/b	Downregulated	Glioblastoma, breast, ovarion and prostate cancer	ERBB2, ERBB3, LIN28, LIN41, TNFSF4	Ectopic expression of miR-125 impairs cell motility and invasion in a breast cancer cell line. Reduction of global miRNA expression enhances migration of cells	170, 259, 322, 327

	Upregulated	Myelodysplastic syndrome, AML with specific translocations and urothelial carcinoma	p53	Ectopic expression of miR-125b suppresses the increase of p53 and stress-induced apoptosis	200
miR-127	Downregulated	Hypermethylation in tumor cell lines	BCL-6, RTL1		345
miR-143, miR-145	Downregulated	Colon adenoma/carcinoma, B cell malignancies, breast, lung and cervical cancer	MYC, ERK5, HOXA9, KRAS, PARP8	miR-143, miR-145 precursor sequences abnormally processed in colon cancer	252, 259, 397
miR-155	Upregulated	Pediatric Burkitt's lymphoma, Hodgkin's lymphoma, primary mediastinal lymphoma, DLBCL, breast, lung, colon and pancreatic cancer	AGTR1, AID, IKBKE, TP53INP1	Pre-B cell proliferation, lymphoblastic leukemia/high-grade lymphoma in miR-155 transgenic mice	170, 199, 200, 397, 388
miR-181	Upregulated	Breast, pancreas, prostate cancer	HOXA11, TCL1	MYCN regulates transcription of miR-181 cluster	388, 390
miR-221/222	Upregulated	CLL, thyroid papillary carcinoma, glioblastoma, (downregulated in AML)	c-KIT, P27, CDKN1B, P57, CDKN1C, ESR1	Promotes cancer cell proliferation; miR-221, miR-222 impair TRAIL-dependent response	388, 398, 399, 400,
miR-200 family	Downregulated	Clear-cell carcinoma, metastatic breast cancer	ZEB1/2, TGF- β	Promote invasion together with miR-205. Downregulation of miR-200 family (and miR-205) directly involved in TGF β -mediated EMT	349, 401
miR-372/373	Upregulated	Testicular cancer (only miR-373)	LATS2, CD44	Indirectly antagonize p53-mediated CDK inhibition during RAS-induced senescence. miR-373 transactivates CDH1 transcription by targeting the promoter region	359, 402

MicroRNAs and breast cancer

Normal breast development

To understand the biological processes underlying human breast cancer, it is important to understand normal breast tissue development. As it is ethical problematic to use human fetal tissues for *in vivo* testing, much knowledge about normal breast cell development comes from mice studies. It has been shown that mammary gland development in mice begins with the formation of fetal lens-shaped placodes that gradually form into a mammary ductal structure, also named an epithelial bud. After twelve days, the bud already consists of multiple epithelial cell layers, surrounded by mammary mesenchyme.²⁷⁰⁻²⁷² In males, further mammary gland development is inhibited by androgens, produced by the testes. In females, each bud starts to elongate to form a primary sprout. Outgrowth of the primary bud is induced by different developmental signals, including interactions between the parathyroid hormone-related protein (PTHrP) and bone morphogenetic protein (BMP).²⁷³ Subsequently, the primary sprout starts to penetrate the fat pad and a hollow lumen is formed. Primary ducts consisting of two epidermal layers develop secondary ducts consisting of one epidermal layer. In total, humans approximately produce ten to twenty ducts. The ductal tree remains in the fat pad, consisting of a central and a basal layer surrounded by mesenchyme, which contributes to the formation of the nipple, also in male (figure 9).^{270,274}

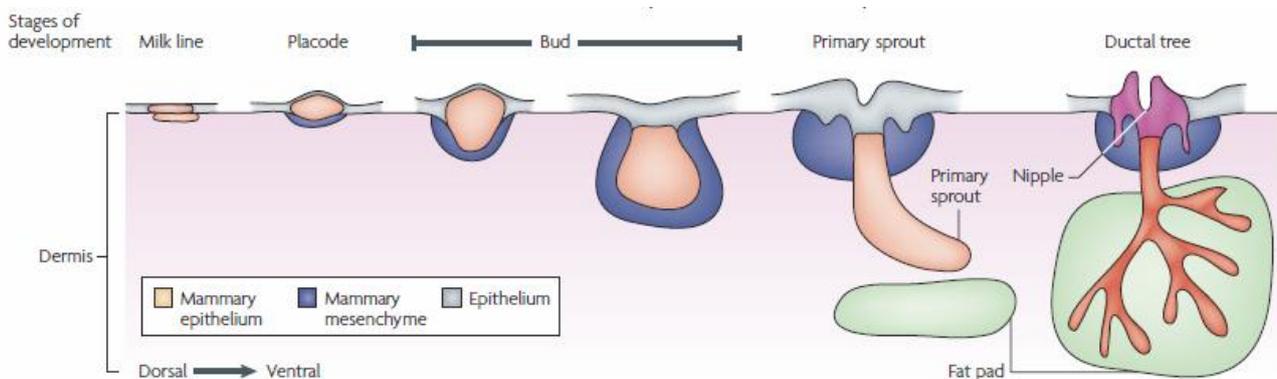


Figure 9. Stages of prenatal breast development. During fetal stages, placodes consisting of mammary epithelium give rise to an epithelial bud, surrounded by mesenchyme. Mesenchym-epithelial interactions induce the primary sprout to invade the fat pad and form a ductal tree. The nipple is connected to the ducts, which is formed by the mesenchyme. The ductal tree remains in the fat pad with low activity, until puberty.²⁷⁵

At puberty, hormones are secreted by the ovaries or pituitary gland to initiate ductal elongation. At ductal elongation, terminal end buds (TEBs) appear at the tips of the ducts that proliferate and continue to invade the fat pad.²⁷² TEB stem cells are thought to give rise to a myoepithelial lineage and a luminal epithelial lineage. The ducts are surrounded by extracellular matrix (ECM) proteins

that must be degraded to enable its movement through the fat pad. The myoepithelial cells are in contact with the basement membrane and surround luminal epithelial cells. These cells do not only play a role in lactation, contracting milk out of the ducts, but are also thought to regulate luminal epithelial cell polarity, branching and differentiation.^{276,277} Lumen formation in the ducts may be developed as a result of cell death in the core.²⁷⁸ The epithelial bud continues to grow and branches into ducts until TEBs have reached the margins of the fat pad.²⁷² Upon cessation of mitotic activity, the terminal end buds regress to blunt-ended ductal structures with a single luminal epithelial cell layer. The differentiated luminal epithelial cell layer is surrounded by a layer of myoepithelial cells (figure 10).

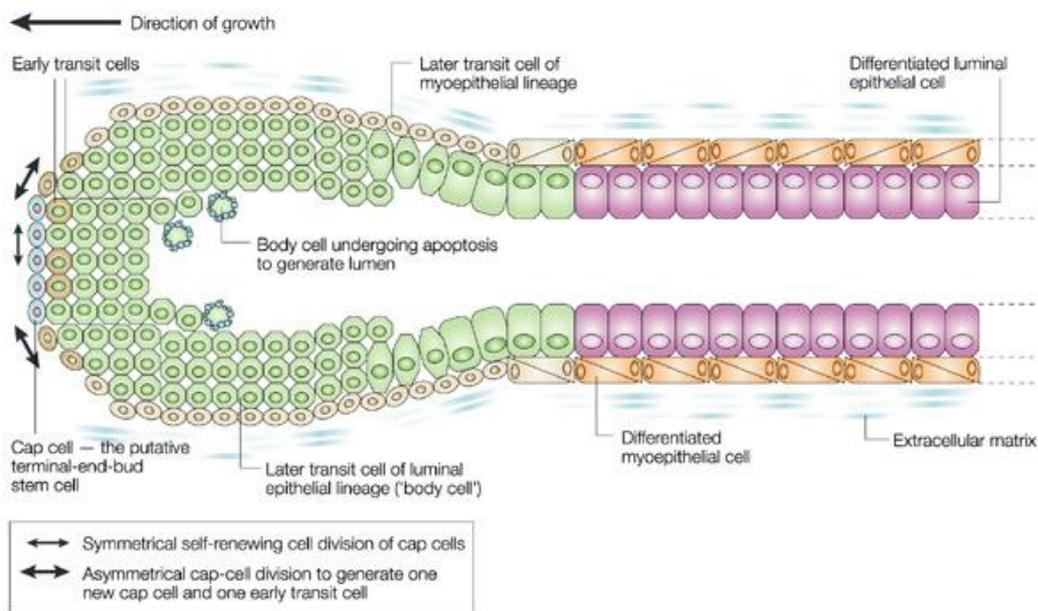


Figure 10. Developing mammary duct. Outgrowth of the epithelial bud into the fat pad is mediated by proliferation of terminal end bud (TEB) stem cells. It is thought that the TEB stem cells generate transit cells of a myoepithelial lineage and a luminal epithelial lineage. The inner cell mass that consists of body cells (transit cells) undergoes apoptosis.²⁷⁹

The ovarian hormones estradiol and progesterone are key regulators of mammary gland development. The majority of proliferating cells are found in the luminal epithelia of the breast. Likewise, estrogen and progesterone receptors are exclusively found in the luminal epithelium. After puberty, hormones regulate the formation of alveolar buds. However, full development of the alveoli and maturation of the mammary gland takes place during pregnancy.^{280,281}

In pregnant mice, circulating levels of progesterone and prolactin induce an increase of ductal branching, expansion of the epithelium, alignment of alveoli buds along the ducts in the fat pad, and, in later pregnancy phases, the formation of large lobular units.^{282,283} The mammary glands are composed of parenchyma, making up the ductular-lobular-alveolar structures. These structures are surrounded by stroma, containing connective tissue, adipose tissue, blood vessels, nerves and lymphatics.²⁸⁴ Luminal epithelial (secretory) cells are surrounded by myoepithelial cells and push

milk out of alveoli spaces upon contraction during lactation. Myoepithelial cells are in their turn connected to a basement membrane.^{281,285} At the end of pregnancy, luminal spaces are filled with fat and milk proteins so that the mother can feed her child. Pregnancy-induced levels of plasma progesterone facilitate milk secretion. When suckling stops, massive apoptosis of the secretory epithelial cells brings the mammary structure back to the adult pre-pregnancy state in a process called involution. Although data is limited, this process is thought to be similar in rodents versus humans (figure 11).^{286 280,283,287,288}

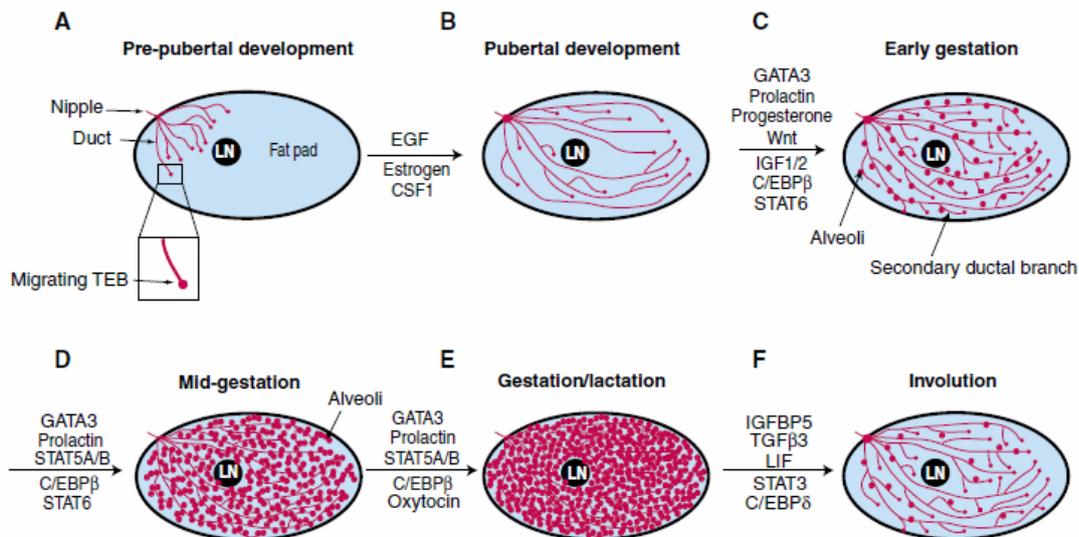


Figure 11. Adult mammary gland development in mice. Before puberty, the ducts move through the fat pad via proliferation and migration of TEB cells. At puberty, ducts continue to grow and branch until they have reached the margins of the fat pad. During pregnancy, branching of the ducts increases and alveoli are formed which eventually leads to the generation of large lobular units. Under influence of hormones, this process continues until breast feeding stops. In a process called involution, apoptosis brings the mammary gland back to a pre-pregnancy state.²⁷²

Breast cancer development

Breast cancer is the most prevalent type of cancer and a major cause of mortality in women worldwide, particularly in North America and Western Europe.²⁸⁹ It is a highly heterogeneous disease, both molecular as clinical. As breast cancer most commonly originates from ductal epithelium, pathological and clinical progression generally starts with ductal hyperproliferation, followed by in situ breast cancer, and may finally progress into metastatic disease. However, although extensively studied, the cell origin of breast carcinoma is unknown. In situ carcinoma is confined to the glandular component of breast tissue, containing an outer myoepithelial layer and an intact basement membrane. Ductal carcinoma in situ (DCIS) accounts for 15-25% of all identified breast cancers. Lobular carcinoma in situ (LCIS) occurs when abnormal cells accumulate in the breast lobules and accounts for 0.5 percent of symptomatic and 1 percent of screen detected cancers.^{290,291} In situ carcinomas have the potential to become invasive, which accounts for about 80% of all cases. Infiltrating ductal carcinoma (IDC) accounts for up to 80% of invasive breast

cancers, while infiltrating lobular carcinoma (ILC) accounts for 10 to 15% of all invasive breast tumors.^{285,291}

Ductal hyperproliferation may start in response to epigenetically and phenotypically changes. Mutations may be familial hereditary, including mutations in *BRCA1* and *BRCA2*, or acquired throughout life (somatic), which is the sporadic variant. The sporadic variant accounts for approximately 90% of breast cancer patients.²⁹² Common gene alterations in breast cancer are amplification of a number of oncogenes and inactivation of some tumor suppressor genes. The most common types of genetic abnormalities in breast tumors are amplification of proto-oncogenes (*Myc*, *ERBB2*) and DNA from chromosome band 11q13; mutation of *TP53*; and loss of heterozygosity from chromosomes and chromosome arms 1, 3p, 6q, 7q, 8p, 11, 13q, 16q, 17, 18q, and 22q.²⁹³

Although the transition of DCIS to invasive carcinoma is poorly understood, the ECM and stromal cells surrounding the mammary gland (including myoepithelial cells and fibroblast) are increasingly recognized to be involved in tumor formation.^{294,295} Stromal cells are abundantly present in breast cancer. Myoepithelial cells secrete protease inhibitors and downregulate matrix metalloprotease (MMP) levels, thereby controlling tumor growth, invasion and angiogenesis.²⁹⁶⁻²⁹⁸ Molecular studies found that myoepithelial cells associated with DCIS have an abnormal phenotype, showing the absence of some differentiation markers, and upregulation of genes promoting angiogenesis and invasion. Moreover, during the transition from in situ to invasive carcinoma, myoepithelial cells were progressively lost.^{296,297} If myoepithelial cells and the basement membrane are lost, the tumor cells may intrude surrounding tissues, becoming invasive.²⁹⁹ This result is in agreement with the hypothesis that fully differentiated myoepithelial cells are natural tumor suppressors.²⁷⁷ If they are able to migrate throughout the surrounding tissues, they may metastasize via the blood stream or via the lymphatic system to distant organs.^{296,300,301}

Another mechanism which is thought to be involved in the invasiveness of breast cancer is the epithelial-mesenchymal transition (EMT). EMT plays a central role in embryogenesis, as this process generates mesodermal cells which can give rise to epidermal organs and individual migratory cells.^{302,303} EMT is characterized by loss of cell-to-cell junctions, reorganization of the cytoskeleton and a spindle-shape with end-to-end polarity.³⁰⁴ This transformation is associated with decreased epithelial specific gene expression, such as E-cadherin, and enhanced mesenchymal-specific gene expression, such as vimentin.^{302,305} Components of the tumor microenvironment, such as metalloproteinases and growth factors, have the ability to induce EMT.³⁰⁴ In addition to being proto-oncogenes, Snail, ZEB (zinc finger E-box binding protein) and Twist are also key transcription factors involved in developmental EMT.³⁰⁶ Artificial expression of *Snail* and *Twist* in epithelial tumor cells induces a transition to a mesenchymal phenotype, which gains malignant properties such as invasion and metastasis.³⁰⁷ Recently, it has been shown that TGF- β can induce EMT, although an invasive phenotype requires an activated Ras in coordination with TGF- β -induced EMT.³⁰⁸ These studies implicate an important role of EMT in tumorigenesis.

One of the best markers that EMT is associated with breast cancer is the loss of E-cadherin, which is partially controlled by the Snail-family. Multiple studies found a partial or complete loss of E-cadherin in breast carcinoma progression.^{309,310} This and other increasing evidence implicate that EMT is involved in the progression of carcinoma in situ to invasive breast cancer.³⁰³

When isolated epithelial cells from different regions in the mammary gland are transplanted into cleared mammary fat pads of mice, new functional terminal duct lobular units can be generated. This experiment suggests the existence of mammary stem cells.³¹¹ Most of the stem cells are located in TEBs and alveolar bud structures during pubertal development in rodents.³¹² The mammary stem cells that have been found in solid breast tumors, exhibit a CD44 high/CD24 low phenotype.³¹³ If tumors become invasive, disseminated cancer cells require a self-renewal capability, which is often enabled by EMT. This raised the question whether EMT also possess a self-renewal capability contributing to the formation of invasive cancer cells.³¹⁴ Indeed, it has been shown that cells that have undergone EMT show some of the same characteristics as normal or neoplastic cell populations.³¹⁵ The regulation of the self-renewal and differentiation of breast stem cells are associated with the Hedgehog pathway, which is involved in embryogenesis and the maintenance and regeneration of adult tissues. *Bmi-1* is one of the downstream targets in the Hedgehog pathway, which is significantly upregulated during activation of the pathway.^{316,317} In addition, its expression was upregulated up to five times in the CD44 high/CD24 low stem cells, compared to cells negative for the stem cell marker.³¹⁷ These results, and numerous other studies, strongly suggest the existence of cancer stem cells and that *Bmi-1* plays an important role in the self-renewal and differentiation of these stem cells.³¹⁸⁻³²¹

Identification of altered miR expression

A growing list of reports suggests that miRs play an important role in breast cancer. The identification of altered miR expression in breast cancer was initially discovered by Iorio *et al*, who identified 29 miRs with aberrant expression patterns in breast cancer tissue compared to normal breast tissue.²⁵⁹ Moreover, 15 miRs could fully discriminate between normal and tumor tissues. The most significantly deregulated miRs were miR-10b, miR-125b, miR-145 (that were down-regulated) and miR-21 and miR-155 (that were up-regulated).²⁵⁹ miR-125b, a homologue of lin-4 in *C. elegans*, was induced in differentiating embryonic stem cells *in vitro* and highly expressed in differentiating tissues.³²² This study implicates the involvement of miR-125b in differentiation, and suggests that their reduced expression in tumor cells could be associated with a poor differentiation. The role and targets of miR-10b, miR-21, miR-145 and miR-155 were at the time of this study only predicted, but not confirmed. Later, investigation confirmed the tumor suppressive role of miR-125b and additionally revealed a tumor suppressive role of miR-145. An oncogenic role was attributed to miR-10b, miR-21 and miR-155 in breast cancer.^{112,323,324}

Since the discovery of altered miR expression in breast tumors, miRs were investigated for their involvement in breast tumor initiation, progression and/or metastasis. Some miRs are identified as playing a role in apoptosis or in cell cycle regulation, while others have been implicated in EMT

regulation or metastasis. Although evidence supported the involvement of miRs in tumor angiogenesis, specific data of miRs in breast cancer angiogenesis is lacking. MiRs that are experimentally identified as playing a role in breast cancer formation, progression and/or metastasis are summarized in table 5.

Altered miR expression in apoptosis and cell cycle regulation

In normal breast tissue, human epidermal growth factor receptor (HER, or also termed ErbB) genes produce growth factor-like receptors on the cell surface. Activation of the HER pathway stimulates its intrinsic intracellular protein tyrosine kinase activity, leading to DNA synthesis and cell proliferation. 15 to 20 percent of breast cancers make an excess of HER2, due to a gene mutation.³²⁵ The activation of HER2 is dependent on interactions with other HER family members. Moreover, the activation of the PI3K/Akt survival pathway is mainly driven through phosphorylation of HER3. MiR-205 targets HER3 receptor, thereby inhibiting HER-mediated proliferation.³²⁶ In addition, miR-125a/b targets both HER2 and HER3.^{102,327} miR-125b was found to be downregulated in human breast cancer.²⁵⁹ In addition, in breast cancer cells overexpressing the HER2 gene product, overexpression of miR-125a or miR-125b impaired growth, migration and invasion capacities of these cells, suggesting a role of miR-125a/b in breast tumor metastasis.³²⁷

One genomic region that is often lost in cancer includes chromosome 13q31. Mapping loss of heterozygosity at chromosome 13q revealed an association of 13q31 with breast tumor progression and poor prognosis.³²⁸ In later research it was found that chromosome 13q31 contains miR-17-5p and that the expression of miR-17-5p was low in breast cancer cell lines.³²⁹ Targets of miR-17-5p include AIB1 (amplified in breast cancer 1), and cell cycle regulators E2F1 and cyclin D1. AIB1 encodes a steroid receptor coactivator that enhances the transcriptional activity of other transcription factor genes, including estrogen receptor α (ER α) and E2F1. The presence of ER α is a risk factor for breast cancer development. Downregulation of AIB1 by miR-17-5p suppresses estrogen-dependent and estrogen-independent breast cancer cell proliferation.³²⁸⁻³³⁰ Decreased expression of miR-17-5p thus contributes to the proliferation of breast cancer cells.

Another miR family that contributes to cell survival in breast cancer is let-7. Let-7 is poorly expressed or deleted in many human cancers, including breast cancer.²⁴⁹ This family targets HMGA2 and cell proliferation factor Ras in breast cancer cell lines. Decreased expression of Let-7 contributes to cell survival.^{110,193,331}

The importance of the Ras signaling pathway in breast tumor cell survival is also evidenced by miR-34a, which targets Ras stabilizing factor *Myc*. *Myc* can drive cell growth, for example via activation of E2F3, and inhibits cell differentiation. In a breast cancer cell line ectopically expressing miR-34a, a small, but significant, increase in apoptosis was shown.³³² MiR-34a is transcriptionally regulated by p53 and was found to be lower expressed in triple negative (both estrogen and progesterone receptor as well as HER2 negative) and mesenchymal breast cancer cell lines compared to normal breast epithelial and HER2 positive cell lines.³³³ The difference in expression might be explained by

p53 mutations in these cell lines.³³⁴ To investigate this, radiosensitivity was tested between normal breast epithelial, mesenchymal and HER2 positive cell lines *in vitro*.³³³ The mesenchymal cell line (with low miR-34a levels) was found to be significantly more sensitive to radiation compared to the other cell lines (with high miR-34a levels). Similar results have been found *in vivo*, in *C. elegans*. These observations indicate that miR-34a is required for survival of mesenchymal breast cells from radiation-induced cell death. In agreement, antagonizing miR-34a increases the radiosensitivity of breast cancer cells.³³³

In addition to the HER signaling pathway, the anti-apoptotic Rhotekin (RTKN) pathway is also regulated by miRs in breast cancer. RTKN is a scaffold protein that links the Rho signal to nuclear factor- κ B, transactivating antiapoptotic genes and inducing cell survival.^{335,336} miR-145 targets the 3'UTR of the RTKN gene, and its increased expression repressed the cellular mRNA and protein levels of RTKN. MiR-145 was down-regulated in human cancer cell line MCF-7 compared to normal breast epithelial cells, contributing to cell growth.²⁵⁸

In contrast to these downregulated tumor suppressor miRs in breast cancer, the oncogenic miR-21 was shown to be highly upregulated in many tumors compared to normal tissue, including breast cancer.^{250,259,337} Studies in mouse xenografts and cell culture revealed that inhibition of miR-21 expression improved cell survival.²⁵⁰ Upon treatment of transfected breast cancer cells and transgenic mice with anti-miR-21 oligonucleotides in combination with an anticancer drug (topotecan), tumor cells became more sensitive to the anticancer drug.²⁵⁰ The increased cell death was associated with downregulation of *BCL2* expression, suggesting that *BCL2* is a target gene of miR-21. *BCL2* mRNA level was decreased in the anti-miR-21 treated cells, suggesting an indirect regulation of miR-21. In further analyses, *PTEN*, *TPM1*, *PDCD4* and serpin and pro-apoptotic gene *Maspin* were identified as direct targets of miR-21, preventing apoptosis and inducing cell growth.^{323,338} *Maspin*, *TPM1* and *PDCD4* also reduced invasiveness of a human hormone-independent metastatic breast cancer cell line (MDA-MB-231).^{323,338} These findings further establish miR-21 as an oncogenic miR, regulating both tumor growth as well as invasion and tumor metastasis.

Another way to induce cell growth in breast cancer cell is by activating the ER signaling pathway. ERs have a DNA binding domain and are involved in regulating gene expression. ER α is overexpressed in approximately 75% of primary breast cancers and is thought to be involved in initiating breast tumors.³³⁹ Binding of estrogen to the ER stimulates proliferation in mammary cells, and increases the chance of getting DNA mutations. Secondly, estrogen metabolism produces genotoxic waste which can also disrupt DNA and influence the cell cycle.³⁴⁰

It was reported that miR-206 was upregulated in ER α -negative breast tumors, suggesting a role of miR-206 in regulation of the ER α gene (*ESR1*).²⁵⁹ Indeed, *ESR1* contained two binding sites for miR-206 in the 3'UTR. MiR-206 expression was higher in ER α -negative breast cancer cells than in ER α -positive cells, and was strongly inhibited by ER α agonists. MiR-206 overexpression reduces ER α levels in human breast adenocarcinoma cells (MCF7), suggesting a negative feedback loop between ER α and miR-206.³⁴¹ Another study showed that miR-206 suppresses *ESR1* expression

and reduces growth of breast cancer cells (MCF9). ESR1 mRNA was also identified as a target of miR-18a/b, miR-193b and miR-302c in breast tumor cells.³⁴² Thus, the ER signaling pathway is, in addition to miR-17-5p targeting AIB1, also regulated by miR-206 in ER α -positive breast cancer.

A very recent study analyzed single-nucleotide polymorphisms (SNPs) in the 3'UTR miR binding sites of various genes. S-phase progression gene *SET8* methylates *TP53* and regulates genome stability. *SET8* contains the 3'UTR binding site for miR-502, and polymorphisms in this binding site may therefore contribute to the early onset of breast cancer. Indeed, data suggested that SNPs in the miR-502 binding site of *SET8* modulates *SET8* expression and contributes, together or independently with the *TP53* SNP, to the early development of breast cancer. However, more and larger studies are necessary to validate these findings.³⁴³

miRs involved in EMT and metastasis

Invasion and metastasis are characteristics of more aggressive or malignant breast cancers. In the recent years, it has become evident that EMT promotes tumor cell motility and invasiveness. The first evidence that miRs were involved in tumor metastases was provided by the group of Weinberg and Ma.³²⁴ They found that miR-10b was highly expressed in many tumors, including pancreatic adenocarcinomas and glioblastomas, and in solely metastasized breast cancer cells.^{266,324} *In vitro* analysis of antisense oligonucleotides that silenced miR-10b led to a significant reduction of the invasive potential of tumor cells, but did not affect cell motility. Subsequent *in vivo* experiments revealed that overexpression of miR-10b in otherwise non-metastatic human breast cancer cells, resulted in muscular and vascular invasion and exhibited high levels of proliferation and angiogenesis. Silencing of miR-10b inhibited Twist-mediated cell migration and invasion.³²⁴ Twist is a transcriptional repressor involved in EMT and a direct transcriptional regulator of miR-10b.^{314,324,344} Subsequent analysis identified HOXD10, a transcription factor involved in development and differentiation, as a direct target of miR-10b. MiR10b was shown to inhibit HOXD10 protein synthesis, which resulted in the upregulation of Ras homologue gene family member C (*RHOC*). *RHOC* is an extensively researched prometastatic gene.^{324,324} Notably, recent investigation reported that targeting of miR-10b in mice appeared to be a successful strategy to reduce metastasis of mammary tumor cells to the lungs. In this study, miR-10b levels were significantly reduced in the antagomir-treated mice, while HOXD10 expression was significantly upregulated. The miR-10b antagomir provides promising evidence that antagomirs can be efficiently delivered to rapidly growing tumor cells *in vivo*, preventing metastasis.³⁴⁵

Two of the *ZFH* (zinc finger homeobox) family members, ZEB1 and ZEB2, are activators of EMT in human cancers by binding to E-box elements upstream of genes and repressing their transcription.³⁴⁶⁻³⁴⁸ The miR-200 family (including miR-200b, miR-200c and miR-429) were shown to target ZEB1 and ZEB2, inhibiting EMT and induce tumors with an epithelial phenotype through inhibition of E-cadherin expression.^{346,349} In agreement with these findings, it was shown that all members of the miR-200 family were downregulated upon growth factor induced EMT in human Madin Darby canine kidney (MDCK) epithelial cells.³⁴⁹ Inhibition of family member miR-200a, b and

c induced growth-factor independent EMT and downregulation of E-cadherin, while overexpression of these family members in mesenchymal cells induced mesenchymal-to-epithelial transition (MET).³⁴⁹⁻³⁵² A recent study identified *Akt2*, an oncogene belonging to the family of serine/threonine kinases, as a miR-200 repressor. This implicates that the *Akt2* controls the miR-200 E-cadherin pathway, thereby controlling metastatic potential of breast cancer cells.³⁴⁶

A few studies found that induction of EMT *in vitro* and *in vivo* resulted in the generation of cells with breast cancer stem cell (bCSC) properties, which suggests that breast epithelial cells can gain bCSC properties via the EMT process.^{311,353,354} Profiling of miR expression in breast cancer stem cells (bCSCs) isolated from human breast tumors, compared to the remaining breast cancer cells, revealed high levels of miR-155 and low miR-200 levels in bCSCs.³⁵⁵

Bmi-1, an important regulator in the self-renewal and in differentiation of mammary stem cells, is another target of miR-200. The miR-200 cluster was downregulated in human bCSCs, normal human and mouse mammary stem/progenitor cells, as well as in embryonal carcinoma cells. Ectopic overexpression of miR-200c in embryonal carcinoma cells stopped cell growth and induced neural differentiation, thereby reduced tumorigenicity of breast cancer stem cells *in vivo*.^{355,356} Thus, the miR-200 cluster is an important regulator of breast tumor progression, metastasis and cancer stem cell differentiation.

As the TGF- β signaling pathway plays an important role in EMT and tumorigenesis, studies also examined the role of miRs in TGF- β induced EMT. In contrast to the tumor suppressive role of miR-200, miR-155 functions as an oncogene and is upregulated during TGF- β induced EMT in mammary epithelial cells. Ectopic expression of miR-155 sensitized the cells to TGF- β and loss of miR-155 suppressed TGF- β induced EMT in normal mouse mammary epithelial cells.³⁵⁷ miR-155 induces metastasis via TGF- β through targeting RhoA. RhoA is a member of the Ras homolog gene family that regulates and stabilizes the formation of cell junctions and stress fibers.³⁵⁸

Other miRs have been identified with a link between metastasis and EMT. For example, Tavazoie *et al.* identified miR-335 as a suppressor of metastasis and migration by targeting transcription factor SRY-related HMG-box 4 (SOX4) and ECM component Tensin C.²⁶⁶ Furthermore, miR-206 and miR-126 are demonstrated to be downregulated in metastatic breast cancer cells compared to parental cells. Restoring miR-206 expression altered cellular morphology, thereby contributing to a decrease in cell motility. It has recently been found that miR-206 expression is increased in ER α -negative tumors and inhibits proliferation of ER α -positive cells.⁴⁰⁷ Mir-126 inhibits cell cycle transition from G1/G0 to S and targets insulin receptor substrate-1 (IRS-1) in breast cancer cell lines.⁴⁰⁴ Huang *et al.* identified miR-373 and miR-520c as inducers of cancer cell migration and invasion, by targeting CD44, which is a metastasis suppressor and a marker of human breast tumor-initiating cells.³⁵⁹ Coupled to increased c-Myc, miR-17-92 expression is increased in metastatic breast cancer cells *in vitro* and *in vivo*.³⁶⁰ However, its target(s) have not yet been identified.²⁶⁶

These studies together support the role of miRs as inducers and inhibitors of breast cancer metastasis (summarized in table 5). However, most of the studies that examined miRs involved in

metastasis were *in vitro*. In future, *in vivo* studies of miRs involved in breast cancer will be important.

Table 5. Experimentally identified miRs in breast cancer.

miR	Tumor suppressor/ oncogene	Target	Cellular function	References
miR-661	Oncogene	Nectin-1, Star-D16	Required for efficient invasion of breast cancer cells.	410
miR-373/520c	Oncogene	CD44	Metastasis.	359
miR-221/222	Oncogene	ER α	Highly expressed in tamoxifen-resistant breast cancer cells. Play a role in the development of ER α negative tumors from ER α positive precursors <i>in vitro</i> . MiR-221 reduced the expression of various tumor suppressors.	407
miR-204 and miR-510	Oncogene	PDEF	Overexpression leads to metastasis progression.	411
miR-103/107	Oncogene	Dicer	High levels of miR-103/107 was associated with metastasis.	409
miR-92	Oncogene	ER β 1	ER β 1 is downregulated in many breast cancers. Inhibition of miR-92 in MCF-7 cells increased ER β 1 expression. The exact function of ER β 1 is not clear yet, but is probably a tumor suppressor.	408
miR-155	Oncogene	RHOA	TGF- β signaling.	357
miR-21	Oncogene	PTEN, TPM1, PDCD4, Maspin, BCL2	Invasion and metastasis: PTEN limits the activity of PI3K pathway. TPM1 regulates microfilament organisation and anchorage independent growth. PDCD4 interacts with EIF4A and prevents protein synthesis. Both PDCD4 and maspin have been implicated in apoptosis and the regulation of uPAR.	323, 338
miR-10b	Oncogene	HOXD10	Invasion and metastasis: miR-10b inhibits synthesis of HOXD10 protein, resulting in upregulation of RHOC which favours cancer cell migration and invasion.	324
miR-9	Oncogene	CDH1	Increased cell motility and invasion.	406
miR-27a	Oncogene	Zinc finger, ZBTB10, Myt-1	Cell cycle progression (G2-M checkpoint regulation).	405
miR-30	Tumor suppressor	Ubc9, ITG β 3	Reduced expression in breast tumor-initiating cells. Constitutive expression of miR-30 in breast tumor initiating cells inhibits their self-renewal capacity. Blocking enhances tumorigenesis and metastasis.	414
miR-17-5p	Tumor suppressor	AIB1, cyclinD, E2F	Oestrogen and E2F1-mediated growth.	329, 330
miR-193b	Tumor suppressor	uPA, ER α	Downregulation of miR-193b leads to tumor progression and invasion.	
miR-18a/b	Tumor suppressor	ER α	Inhibition of ER-mediated cell growth.	412, 413

miR-206	Tumor suppressor	ER α	Oestrogen-mediated growth.	259, 341, 407
miR-335	Tumor suppressor	SOX4, PTPRN2, MERTK, TNC	Metastasis.	266
miR-27b	Tumor suppressor	CYP1B1	Modulation of the response of tumours to anti-cancer drugs.	403
miR-125a/b	Tumor suppressor	HER-2/3	Anchorage-dependent growth. Migration and invasion.	102, 112
miR-200	Tumor suppressor	TCF-8, ZEB1/2, BMI1	TGF- β signaling. EMT and initiation of an invasive phenotype (miR-200c).	346,349, 355
miR-126	Tumor suppressor	IRS-1	Cell cycle progression from G1/G0 to S.	404
miR-145	Tumor suppressor	RTNK	Inhibits breast tumor growth.	210
miR-146a/b	Tumor suppressor	EGFR	Metastasis suppressor <i>in vitro</i> .	412
miR-205	Tumor suppressor	HER-3	Inhibiting HER-mediated proliferative signaling.	326
miR-34a	Tumor suppressor	CCND1, CDK6, E2F3, MYC	DNA damage, proliferation.	176, 334
miR-31	Tumor suppressor	F2D3, ITGA5, M-RIP, MMP16, RDX, RHOA	Metastasis.	383
Let-7	Tumor suppressor	H-RAS, HMGA2, Lin28, PEBP1	Self-renewal and differentiation of T-IC.	83, 193, 110

Discussion

Breast cancer is the most prevalent type of cancer and the second leading cause of cancer deaths in women worldwide. Breast cell malignancies are highly heterogeneous, differing in their cell of origin, the molecular pathway underlying the cause, as well as in susceptibility and defense between individuals. Unfortunately, the molecular mechanisms underlying breast cancer initiation, progression and metastasis remain poorly understood. Therefore, the need for good prognostic indicators and better therapeutic interventions increases. MiRs are suggested to be potential candidates as diagnostic and prognostic indicators, and are currently also tested as therapeutic candidates in the fight against cancer.

Although the miR story has just started, increasing evidence suggest that these molecules are involved in various physiological processes and human pathologies, discussed in this paper. They are strongly evolutionarily conserved throughout metazoans, implicating their importance. MiRs regulate genes at posttranscriptional level by binding with their seed sequences to complementary sites of the mRNA 3'UTR, leading to mRNA degradation or translational repression. In human cancers, the genomic location in which miRs reside may be lost, amplified or situated at breakpoint regions of translocations.¹⁰⁸ As summarized in this review, altered miR expression has been observed in breast cancer related to apoptosis, cell proliferation and metastasis. However, whether altered expression of miRs in these processes is a consequence of increasing DNA damage, or an initiating event causing it, is not clear yet.

Studying miR expression

As mentioned in this report, miRs may repress or activate their target mRNA in multiple ways. Although much research has been focused on miR target gene regulation, relatively little is known and published about the expression and regulation of miR genes themselves. However, revealing tumor specific miR expression is also important in clarifying the role of miRs in tumorigenesis. MiR expression is considered to be highly specific in tissues and developmental stages.³⁶¹ Recent evidence showed that miR expression in cancer tissue is also specific and may be used for tumor characterization as a biomarker.^{101,337} In human breast cancer for example, miRs may be profiled based on different expression in various breast cancer subtypes. However, in these studies, miR expression in breast cancer tissue was compared to normal breast tissue consisting of adipose tissue. Considering that miR expression may be tissue specific or even tissue-cell specific, the comparison needs to be made with normal mammary epithelial cells.^{259,362} This consideration needs to be taken into account when studying tissue specificity of miR expression.

Studying miR targets

To gain more insights into the working mechanism of miRs, different computational algorithms have been developed to predict miR target genes. Most computational methods search for complementarity in the 3' end of mRNA to seed matches in the 5' end of miR, thermodynamic stability and evolutionary conservation. Although less important, miR 3'-end pairing may also contribute to target recognition, in particular when sites have weaker miR seed matches.³⁶³ In fact,

experiments with artificial sensor construct in human cell lines showed that target sites in 5' UTR of mRNA showed equal levels of repression by miR as sites located in 3' UTR.³⁶⁴ Therefore, by only looking at the 3' UTR of mRNA, complementarities in the 5' end may be overlooked, which results in target overlap. Furthermore, the idea that targets could be predicted based on seed regions, was only introduced because this region was the only contiguous site of miRs that was evolutionarily conserved. However, non-seed target sites have also been discovered, for example lin-4 and lin-7 target sites in lin-41 and lin-14.^{13,93,365-367} So, prediction technique should also be aimed at non-seed target sites and 5' end mRNA regions.

Recently, a new computational architecture, named MTar, includes site complementarity at the 5' end of miR and also takes structural and positional features of miR-mRNA interactions into account.³⁶⁸ Target predictions need to be confirmed by biochemical evidence of changes in corresponding mRNA and/or proteins resulting from experimental alterations of miRs and target sequences. Because many mature miR strands are distinguished by only one or two nucleotides, current methods cannot avoid the possibility of detecting similar miRs. Furthermore, as mature miRs are very small, it makes it difficult to use PCR for measuring differences in expression between different miRs. Thus, improvement of the current techniques in terms of sensitivity and specificity is desirable for better miR target prediction and validation.

Target detection is not feasible, as a genome-wide statistical analysis showed that one miR targets on average hundred evolutionarily conserved target sites.⁸⁵ This indicates that miRs regulate a large fraction of protein-coding genes. More than 40% of human miRs are situated within 10 kb of another miR in the genome.³⁶⁹ Furthermore, intragenic miRs are co-expressed with the mRNAs of host genes, probably sharing the same promoter and expression control elements.¹²¹ Together, these features implicate that downregulation or loss of function of miRs may result in dysregulation of many processes. This is not preferable in designing miRs for therapeutic interventions, as therapeutics should be specifically aimed at cancer cells.

Methods such as RT-PCR, micro-array analysis and target prediction algorithms may identify miR targets, the pathways they regulate, and tumor specific miR expression. As a single miR can target multiple genes, it may be more feasible to target miRs involved in a specific cellular process, even without knowing all the target genes.³⁷⁰ By introducing miR mimics or anti-miRs in tumors with deleted or overexpressed miR genes, apoptosis and/or cell cycle arrest may be induced in tumors that depend on miR dysregulation for growth and survival. MiRs or anti-miRs can be delivered via vectors, such as adenoviruses or retroviruses, or liposomes for tissue specific delivery. As an alternative, miR (anti-) oligonucleotides can be applied directly, without the need of a vector. However, direct delivery of oligonucleotides is not very stable, it may have only a transient effect, and they may require repeated deliveries.¹⁴⁵

Testing miR mimics, anti-miRs and sponges for therapeutic potential

A number of miR mimics and anti-miRs have already been tested in mice for therapeutic potential. For example, restored expression of miR-124 and miR-203 by synthetic miR-oligonucleotides significantly reduced cell proliferation in hepatocellular carcinoma cell lines.³⁷¹

Let-7 introduced into a lung cancer cell lines is an example of a vector-based delivery. Let-7 family members are downregulated in lung cancers both *in vivo* and *in vitro*. Expression constructs were designed to synthesize mature miRs of two predominant let-7 isoforms, let-7a and let-7f under the control of the RNA polymerase-III H1-RNA gene promoter. Overexpression of let-7f and let-7a resulted in growth-inhibitory effects of lung cancer cells.¹⁰⁹ miR-15a and miR-16-1 have also been tested *in vivo*. Transfection of leukemic cells (MEG-01) with miR-15a and miR-16-1 containing plasmid vectors (pRS) inhibited the growth of leukemic cell engraftments in nude mice.³⁷² Several artificial or authentic miRs can be simultaneously expressed by a tandem array on a precursor RNA transcript.³⁷³ In this way, 10 miR mimics directed against several sites within the *ABL* oncogene coding sequences were designed. Chimeric Bcr-Abl gene expression, a specific chromosomal abnormality, is associated with chronic myelogenous leukemia (CML). Comparison between the suppression of each miR mimic revealed that each could effectively suppress the Abl protein through targeting of Abl sequences in an aggressive pre-B leukemia model.³⁷⁴ Such a construct was also used to silence chemokine receptor CXCR4 in human breast adenocarcinoma cells (MDA-231).³⁷⁵ The expression pattern of CXCR4 is significantly correlated with the degree of lymph node metastasis in breast cancers, implicating that artificial miR may potentially be a therapeutic agent to prevent breast cancer metastasis.³⁷⁶

Numerous preclinical studies have shown promising results with using recombinant adeno-associated virus (rAAV) gene delivery vectors.³⁷⁷ However, self-complementary AAV (scAAV) vectors are even more effectively, as they bypass the need to convert single-stranded DNA into double-stranded DNA before expression. For example, an scAAV vector containing miR-26a was injected into mice with established liver tumors. High miR-26a levels were found in their liver and no toxic effects were observed. While 6 out of 8 mice treated with control virus developed tumors, 8 out of 10 'vector treated' mice developed only small tumors or no tumors at all.³⁷⁸ This study shows important potential to use the AAV vector approach as a therapeutic against cancer. Although AAV vectors are currently used in many gene transfer applications, more investigation needs to be done to test other miRs and their therapeutic potential.³⁷⁹⁻³⁸¹

Oligonucleotides can also be modified to improve binding affinity to RNA. The oldest, simplest and most common used modification in this regard is to put a 2'-O-methyl group to oligonucleotides. For example, 2'-O-methyl antisense oligonucleotides were used in a study to knock down miR-125b in a prostate cancer and a cervical cancer cell line. Cell proliferation was reduced in both cell lines.³²² Oligonucleotides can also be modified with a 2'-O-methoxyethyl group to further enhance RNA specificity and affinity, or with incorporation of conformationally locked nucleotide acid (LNA) monomers to increase stability and improve mismatch discrimination.³⁸² This latter method was used to specifically inhibit miR-21 in glioblastoma and breast cancer cells.²⁵³ Anti-miR-21 oligonucleotides were introduced in breast cancer cells which resulted in the suppression of tumor cell growth, both *in vitro* and upon xenografting. Growth suppression was associated with increased apoptosis and decreased cell proliferation, probably through downregulation of *BCL2*.²⁵⁰

As an alternative to chemically modified antisense oligonucleotides (antagomiRs), competitive miR inhibitors are developed that contain multiple binding sites to a miR of interest, preventing it to bind to its natural target. These inhibitors that 'soak up' endogenous miRs, called 'miR sponges',

are transcripts expressed from strong RNA pol II promoters and specifically inhibit miRs with a complementary seed sequence (position 2-8 of the miR). In this way, a single sponge can block all miR family members containing the same seed sequences. For the detection and sorting of sponge-transfected cells, a fluorescence reporter gene is included in the vector.¹⁰⁶

This 'knock-out' method is a more powerful tool to identify miR function compared to the antagomiR approach, because it can be applied in any transgenic model organism (even in plants, flies, and worms), is less time consuming and it can better inhibit functional classes of miRs (in stead of single miR sequences). In addition, because up to 40% of the miR genes are located in protein-coding genes, inhibiting or knockout miRs may create an artifact. The observed result may not be the result of the lost miR, but may be the result of the lost protein.^{106,145}

For example, the sponge strategy has been used to inhibit miR-31 *in vivo* in a noninvasive breast cancer cell line (MCF-7-Ras). MiR sponges that carried miR-31 recognition motifs were introduced into a retroviral vector and reduced miR-31 function significantly. As miR-31 expression correlates inversely with metastasis in human breast cancer patients, the otherwise nonaggressive breast cancer cells were able to metastasize.³⁸³

However, the sponge technology also has limitations. Overexpression of target genes may be toxic for the cells and is not always achieved in certain cells or tissues. Furthermore, there are limitations in the usefulness of sponges *in vivo* for conditional or tissue specific depletion.¹⁴⁵

Recently, Loya *et al.* addressed the tissue-specificity limitation by developing Gal4-regulated gene expression miR sponges. The sponges contain 10 miR target sites with central bulges for an optimal stability, and Gal4-directed expression permits tissue-specific expression. They used their sponge to demonstrate that miR-8 is required in muscle cells to support neuromuscular junction formation.³⁸⁴ This study demonstrates the potential of miR sponges to explore miR functions spatially and temporally *in vivo*.

Future approach: targeting miR expression or specific miR target genes?

Together, these examples show that targeted therapies that induce or inhibit dysregulated miRs may be a promising treatment of human malignancies (summarized in figure 12). Although experiments in mice show modest side effects, the (side)effects of knocking down or overexpressing miRs in humans have not been explored. As a single miR may target hundred genes in humans and the explicit knowledge of individual roles of miRs or miR families in cancer is too limited, the chance of having toxic side effects in humans is too high. However, toxic side effects may be limited when miR targets involved in cancer are altered instead of miR expression. Although miRs target many genes, not all of their target genes are involved in cancer. Therefore, specifically alter miR target genes involved in cancer by using alternative strategies may hold greater potential for anti-cancer therapy than using miR sponges, miR mimics or oncomiRs. For example, the miR-masking strategy utilizes a perfect complementary sequence to the miR target gene, such that it 'competes out' the interaction between a specific miR and its target gene. This gene-specific approach has been tested in a number of miRs in several models.^{415,416}

Simultaneously targeting multiple members of a gene family can also be a useful strategy to interfere with miR target genes. Small multiple target artificial (SMART) miRs are currently developed. They may target, for example, all E2F members.⁴¹⁷

In summary, specificity of miR expression in cells or tissues, and their specificity in target regulation have not yet been confirmed and needs to be further investigated. A modest change of a miR may induce a chain reaction that influences feedback pathways involving various miRs and affecting multiple target genes and pathways. As tumor formation is a multi-step process, therapeutics should initially be aimed at the initiating events. Over the past few years, targeted therapies become more and more important for cancer patients. A future challenge will be to define the exact role and specificity of miRs in tumor initiation, progression and metastasis. Testing the functional roles of miRs, miR families and their long term effects *in vivo* can be achieved by using miR mimics, antagomiRs and sponges. If the exact function of miRs in tumorigenesis has been revealed, targeting cancer-specific miRs or using strategies that specifically alter miR target genes may hold great potential for future therapeutic interventions.

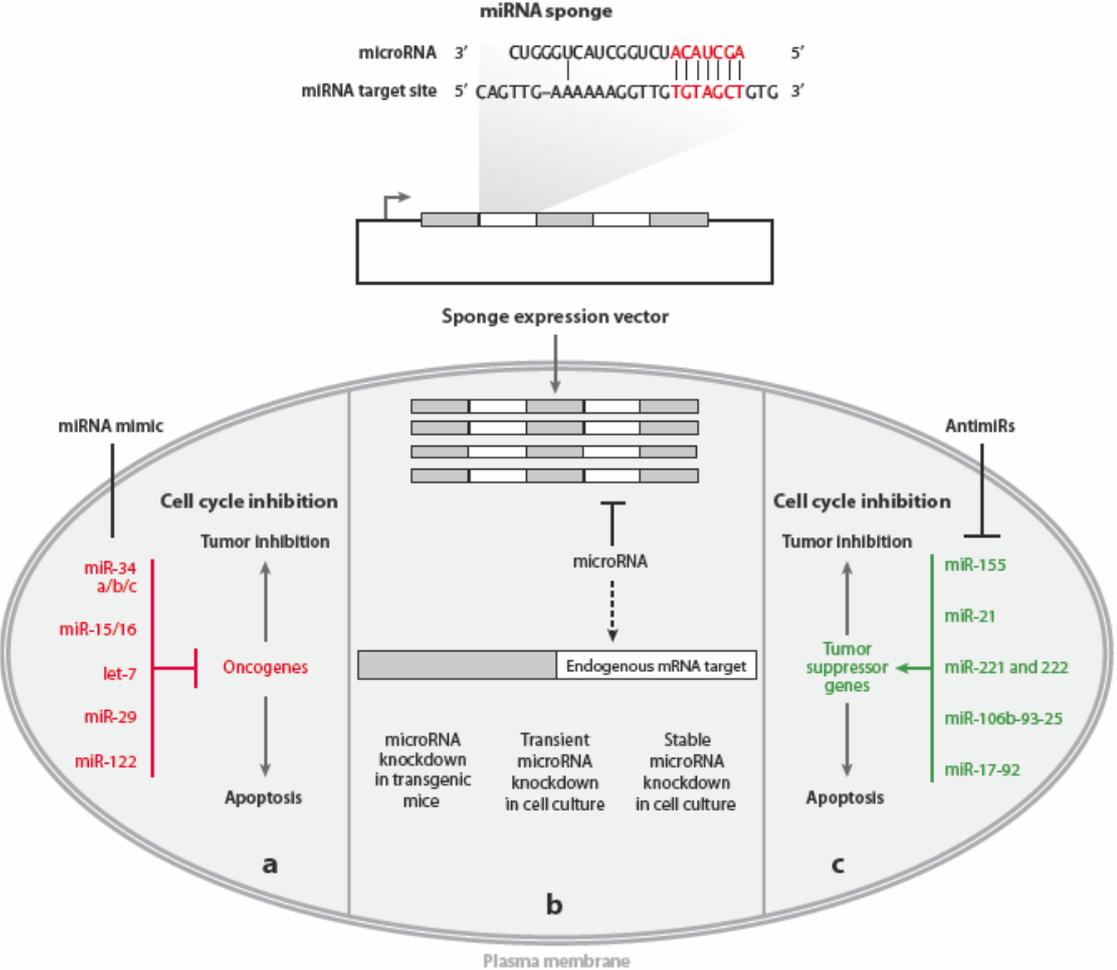


Figure 12. MiR mimics, sponges and antagomiRs. A) The miR mimic technology: synthetic miRs that mimic endogenous (tumor suppressor) miRs bind specifically to their target genes and repress them posttranscriptionally. B) Sponges: ectopically expressed mRNAs that contain multiple binding sites to a miR of interest, preventing it to bind to its natural target. This method is suitable *in vitro*, as well as *in vivo*. C) AntagomiRs: knockdown of miRs through anti-miR oligonucleotides, suitable *in vitro* and *in vivo*. In cancer they may target oncogenic miRs.

References

Additionally used references in tables: ³⁸⁵⁻⁴¹⁷

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