CLASSICS AND NON-CANONICAL FUNCTIONS OF MIRNAS IN CANCERS

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Abstract

Alterations in microRNA (miRNA) expression are causative in initiation and progression of human cancers. The molecular events responsible for the widespread differential expression of miRNAs in malignancy are represented by their location in cancer-associated genomic regions, epigenetic mechanisms, transcriptional dysregulation, chemical modifications and editing, and alterations in miRNA biogenesis proteins. The classical miRNA function is synonymous with post-transcriptional repression of target protein genes. However, several studies have reported miRNAs functioning outside this paradigm and some of these novel modes of regulation of gene expression have been implicated in cancers. Here, we summarize key aspect of miRNA involvement in cancer, with a special focus on these lesser studied mechanisms of action.

Glossary

Argonaute family (AGO): a protein family (AGO 1-4) with important role in RNA mediated silencing. The most important, AGO2, is the key element of the RISC, resulting in endonucleolytic cleavage and degradation of targeted mRNA.

CpG islands: short DNA fragments containing a high GC content. The cytosines in these regions can be methylated to form 5-methylcytosines, affecting the expression of surrounding genes. CpG islands are usually located in promotor and enhancer regions.

DICER1: a cytoplasmic RNase III endonuclease that process, together with TRBP, the pre-miRNA into a 22 nt duplexe with a 2-nt overhang at their 3′ ends, before one of the strands are incorporated into RISC.

DROSHA: RNase III endonuclease that interacts with DGCR8 in the nucleus and is responsible for possessing of pri-miRNA transcripts into a 65-70 nt stem-loop precursor miRNAs.

Epithelial to mesenchymal transition (EMT): is a naturally occurring, transdifferentiation program by which polarized epithelial cells lose their adherent and tight cell–cell junctions, enhance their migratory capacity, and elevate their resistance to apoptosis. The program is important in embryonic development, wound healing, and cancer metastasis.

Exosomes: small vesicles with a lipid bilayer membrane secreted by cells. Exosomes travel in different body fluids, containing various types of cargos, including miRNAs, which is transferred between cells.

IsomiRs: a pri-miRNA can give rise to multiple isoforms of mature miRNAs that have different primary sequence than the original two complementary mature miRNAs (-3p and -5p), and these miRNAs are termed isomiRs. IsomiRs contain either deletions or extensions at the 5′- or 3′-ends, or single nucleotide changes within the miRNA.

MiRNA seed region: nucleotides 2–8 of the mature miRNA. The seed region is mainly responsible for mRNA target recognition. Mutations in the seed region will cause a shift in the targetome.

MiRNA targetome: all RNA targets a miRNA interacts with by complementary binding. Can be both mRNAs and non-coding RNA species.

Oncogenic miRNA (oncomiR): a miRNA that plays a pro-tumorigenic role.

Precursor miRNA transcript (pre-miRNA): a 65-70 nt stem-loop precursor transcript generated after DROSHA cleavage of the pri-miRNA transcript in the nucleus.

Primary miRNA transcript (pri-miRNA): initial RNA transcript made from the miRNA gene, often transcribed by RNA polymerase II, that takes the form of specific hairpin structure, generated after complementary binding of internal regions in the pri-miRNA.

RNA induced silencing complex (RISC): a protein complex with an important role in post-transcriptional gene-silencing using RNA fragments as guides. Key components of RISC are proteins of the Argonaute protein family, especially AGO2.

Single nucleotide polymorphisms (SNPs): a substitution of a single nucleotide in the germline DNA of a large portion of the general population.

Tumor microenvironment (TME): all non-neoplastic cells (immune cells, blood vessels, and fibroblasts), extracellular matrix components, and signaling molecules located near tumor cells.

Tumor suppressor miRNA: a miRNA that inhibits tumorigenesis.

1. Introduction

MicroRNAs (miRNAs), a subclass of small non-coding RNAs (ncRNAs), were initially associated with cancer two decades ago, in 2002 [1], only a decade after their initial discovery [2, 3]. Since then, out of a literature of over 60.000 papers, miRNAs have been proven to drive tumorigenesis [4], to be exploited as biomarkers [5], and to be used for and as novel RNA therapeutics [6]. MiRNAs are single-stranded RNA molecules of approximately 19-24 nucleotides (nt), typically excised from 60- to 110 nt RNA hairpin precursors [7]. MiRNAs are transcribed as primary miRNAs (**pri-miRNAs**, *see Glossary*), which are subsequently cleaved into precursor miRNAs (**pre-miRNAs**) and further processed into mature single stranded ~22 nt miRNAs. The biogenesis of miRNAs involves a complex protein system, including the RNase III enzymes **DROSHA** and **DICER1**, members of the **Argonaute family** (AGO1-4), and Pol II-dependent transcription [8-10]. Global loss of expression of miRNAs through deletion of specific miRNA biogenesis proteins results in early lethality in mice [11], reflecting the importance of miRNA in normal development.

The number of human mature miRNAs reported to date [12] is in excess of 2600, ten times as many as the initial calculations indicated [13]. MiRNAs are involved in critical biological processes, including proliferation, differentiation, and apoptosis [7], and they are expressed in distinct spatial and temporal patterns, both during embryonic and postnatal development and in adult tissues [11]. The classic function of miRNAs is to post-transcriptionally repress expression of specific target proteins by either promoting messenger RNA (mRNA) decay or by dampening translation [7, 14, 15]. A growing number of studies have reported miRNAs functioning outside this paradigm, including translational upregulation, epigenetic regulation, transcriptional activation, as well as their presence in mitochondria and in the nucleus [16].

2. Mechanisms of miRNA dysregulationin cancer

The different expression pattern of miRNAs between cancer cells and normal cells, or between bodily fluids of cancer patients and healthy individuals **(BOX 1)**, is complex and is regulated through several mechanisms like deletions or amplifications of miRNA loci, mutation of *MIRNA* genes, epigenetic and transcriptional regulation, posttranscriptional modification (i.e. editing and chemical modifications), and dysregulation in miRNA processing. The role of miRNAs in

cancer was discovered due to their location in loci that are frequently deleted or amplified in cancer, named cancer associated genomic regions [1]. These include the 13q14 region, which is deleted in over half of patients with the most frequent leukemia in the Western word, the chronic lymphocytic leukemia (CLL), harboring the first discovered cancer-related miRNAs, miR-15a and miR-16-1 [1]. Subsequent genome wide analysis proved that more than half of miRNAs are located at fragile sites, regions of loss of heterozygosity, minimal amplicons, or breakpoint sites in humans [17, 18], mice [18], or canine [19] genomes.

2.1. Germline and somatic mutations of miRNAs

It is widely accepted that cancer is a disease caused mainly by somatic mutations [20]. Mutations of miRNAs can induce a change in the **miRNA targetome** (**Figure 1**), if occurring in the **seed region**, or it can alter the biogenesis by inducing destabilization of the hairpin structure or changing the interaction capacity with regulatory proteins like DROSHA and DICER1 [21-23]. A germline mutation in the *MIR15A*/*MIR16-1* pri-miRNA located 7 bp after the 3'end of *MIR-16-1* was the first ever miRNA mutation discovered in human cancers (specifically in a family in which both CLL and breast cancer were occurring) [24]. It caused low levels of miR-15a-5p and miR-16-5p and was associated with deletion of the normal allele [24], the classic Knudson model of tumorigenesis [25]. A mutation located in a similar position was further identified specifically in the mouse strain that naturally develops a disease similar to CLL [26]. Mechanistically, the mutation is located in the *MIR16-1* CNNC motif and disrupts recruitment of SRp20, a member of the serine/arginine (SR)-rich family of pre-mRNA splicing factors that affects the pri-miRNA processing and lowers miR-15a-5p/16-5p accumulation [21].

Recently, over 10.588 miRNA mutations were discovered by the investigation of over 10.000 cancers from the TCGA repository [27]. Almost one third of patients showed at least one miRNA mutation, with most mutated miRNAs in melanoma, diffuse large B-cell lymphoma (DLBCL), and lung squamous cell carcinoma. MiRNA mutations were equally distributed in all regions of the gene with no positive enrichment of mutations in the seed regions. The most mutated miRNAs in the pan-cancer analysis were *MIR1324*, *MIR1303,* and *MIR4686,* and the most mutated miRNA in a specific cancer was the ultraconserved *MIR142*, harboring driver mutations in DLBCL [27], as well as in CLL [28], acute myeloid leukemia (ALL) [29, 30], and other types of lymphomas [31]. Supporting the driver role of miR-142-5p is the fact that *MIR142*

knock out mice show a profound immunodeficiency characterized by aberrant lymphopoiesis of both B-cells and T-cells [32]. Other miRNAs frequently mutated in the pan-cancer analysis were *MIR205* and *LET7D*, both highly conserved miRNAs [27], proving once more that conservation is a hallmark of functionality.

Not only miRNAs are mutated in cancer but also their mRNA targets, or miRNA sponges (**BOX 2**). These can suffer point mutations or even complex deletions in their 3' UTR miRNA binding sites [33]. The mutational events can lead to loss of complementarity between miRNAs and mRNAs and loss of target inhibition. A notable example is a mutation in the 3'UTR of the oncogene *E2F1* in colorectal cancer, leading to a loss of miR-136–5p target recognition [34]. This mutation induces a 4-fold increase in E2F1 expression, potentially being associated with a more tumorigenic phenotype [34]. Similar mechanisms that can increase the susceptibility for cancer or protect against cancer occurrence by modulating the miRNA target recognition have been described for **single nucleotide polymorphisms** (SNPs) located in the 3'UTR binding site [35].

2.2. Epigenetic regulation

Epigenetic regulation is an essential mechanism of controlling gene expression, and the most studied type of epigenetic regulation is DNA methylation at **CpG islands**. It was considered that CpG islands are overlapping mainly promotor regions of coding genes [36], and only more recently it was observed that CpG islands are located also close to or overlapping with miRNAs. MiRNAs, depending on their genomic location, can be regulated by methylation in several ways (**Figure 1**). Intergenic miRNAs can have a CpG island overlapping their transcriptional start site, similar to coding genes [37], or can have a promotor region with several CpG dinucleotides, but not a full CpG island that controls their expression [38], or can have internal CpG islands and thereby be silenced by their methylation [39]. On the other hand, intragenic miRNAs are often regulated by methylation together with their host genes. However, several studies have revealed that the expression of miRNAs and their host genes does not always correlate [40], indicating that differential expression, maturation, or that the stability of the host gene and the miRNA can differ. This is of clinical relevance, as treatment of cancer cells with DNA-demethylating agents can reactivate the expression of **tumor-suppressive miRNAs**, such as miR-148a-3p, miR-34b-3p, miR-34c-5p, and miR-9-5p [41]. MiRNA expression is also

regulated by post-translational modifications of histones. Several tumor related miRNAs are regulated by these epigenetic changes, especially by lysine acetylation or methylation [42] (see EpimiR in [43]).

2.3. Modulation of the miRNA biogenesis machinery

A global downregulation of miRNAs has been associated with cells undergoing **epithelial to mesenchymal transition** (EMT) and stem cell characteristics [44, 45]. In addition, a general downregulation of miRNAs, because of mutations or dysregulation of components of the miRNA biogenesis pathway, has been reported for multiple cancers [46]. By exploring the TCGA repository, over 3600 somatic mutations in 29 miRNA biogenesis genes were identified with some of these being over-mutated in specific cancers or associated with patient survival [47]. DROSHA and DICER1 are key proteins in the biogenesis of miRNAs, and their expression has been found to be downregulated in several types of cancer (**Figure 1**) [48-50]. Further, germline mutations in proteins involved in miRNA processing and maturation have been associated with increased cancer risk [51, 52]. Heterozygous germline mutations in *DICER1* were identified in families affected by pleuropulmonary blastoma [51]. The majority of the identified mutations resulted in protein truncation proximal to the two carboxy-terminal RNase III functional domains in DICER1, and the authors proposed that loss of DICER1 caused a global reduction in miRNA expression, which further promoted mesenchymal proliferation. In addition, somatic mutation in the RNase III functional domains was identified in ovarian cancer [52]. These mutations did not obliterate DICER1 function, but reduced the RNase IIIb activity of the protein. Knock down of *DROSHA* was sufficient to increase proliferation both *in vitro* and *in vivo* in lung adenocarcinoma cells [53], and *DROSHA* has been found to be frequently mutated in children diagnosed with Wilms tumor [54-56]

The AGO-miRNA complex forms the core of the RNA induced silencing complex **(RISC)**. In humans, four AGO proteins exist (AGO1-AGO4), but only AGO2 harbors nuclease activity. Mechanisms for specific loading of miRNAs into the four distinct AGO proteins are still unknown, and AGO protein expression differs both during embryonic development and across different tissues [57]. AGO1 and AGO2 are the most prominent AGO proteins in normal tissue [57], and a dysregulated expression has been observed in cancer [58]. The AGO proteins are

important for the stability and turnover of miRNAs [59, 60], and for these reasons, their dysregulation will have consequences for the miRNA expression.

Increased expression of the miRNA biogenesis proteins leads to a positive global change in miRNA expression. *DROSHA* copy-number gain or overexpression was found in more than 50% of advanced cervical squamous cell carcinomas [61]. Importantly, DICER1 and DROSHA has been implicated with important cellular mechanisms outside of miRNA maturation [62]. With these findings, phenotypes discovered in knock out mice models might not only be a consequence of global miRNA regulation. In addition, miRNA biogenesis has been found to be regulated by paraspeckles [63] as well as by novel proteins [64]. Further, autophagy has been identified to be important for miRNA turnover [65]. These new regulatory events increase the complexity of miRNA biogenesis and stability.

2.4. Editing and chemical modifications of miRNAs

In the last years novel post-transcriptional regulatory mechanisms of miRNAs, including miRNA editing and chemical modifications (reviewed in [66]) have been characterized and found to play an important role in cancer. The most common editing mechanism of miRNAs is the ADAR dependent adenosine to inosine (A-to-I) editing (**Figure 1**). The editing of both pri- and pre-miRNAs close to the DROSHA/DICER1 recognition sites can impact the miRNA biogenesis [67]. Additionally, editing of the mature miRNA sequence, including the seed region, can cause modified stability and change in its targetome [68]. Nineteen ADAR dependent A-to-I RNA editing hot spots in the mature sequence of miRNAs have been identified [69]. The most edited miRNAs were miR-589-3p, miR-381-3p, and miR-200b-3p. Furthermore, the editing of miR-200b-3p, a tumor suppressor miRNA, transforms the miRNA into an **oncomiR**. Edited miR-200b-3p levels, but not WT miR-200b-3p levels, associate with a shorter overall survival for cancer patients, and at the molecular level, edited miR-200b-3p loses its ability to target the EMT regulators *ZEB1/ZEB2* and suppresses *LIFR*, a metastasis inhibitor [69].

Opposite to RNA editing, chemical modifications of RNAs are reversible and refer to the addition of different chemical groups to the structure of transcribed RNAs, including miRNAs [66]. Multiple types of chemical modifications of miRNAs with oncogenic roles were recently described including: 5-Methylcytosine (m^5C), N^6-Methyladenosine (m^6A), 7-Methylguanosine

(m^7G), pseudouridylation (Ψ), and uridylation [66]. m^6A methylation of miRNAs impairs the ability of miRNAs to downregulate their targets (**Figure 1**) compared to m^5C-methylated or wild type transcript. From a clinical standpoint, analyzing serum derived methylated miR-17-5p and let-7a-5p is more specific and sensitive for the detection of gastrointestinal cancers than the currently available protein-based markers [70]. Recently, it was demonstrated that small RNAs are modified with N-glycans [71, 72]. This open up for a new type of modification of RNAs, and might hold potential for miRNAs to also be modified by glycoproteins and glycolipids.

3. MiRNAs as dual players

3.1 miRNAs as oncogenes and tumor suppressors

miRNAs are involved in the regulation of all cancer hallmarks [73]. While some miRNAs work as archetypal oncogenes (e.g. miR-21 or miR-155) or tumor suppressors (e.g. miR-34a, the miR-miR-15a/16-1 cluster) in cancer [74-77], other miRNAs play context dependent roles in cancers, being in some malignancies oncomiRs, while in others tumor suppressors. One such example is miR-146a-5p, a miRNA that plays an important immunological role and similar to the immune response, in cancer it can be both pro- and anti-tumorigenic. Knock out of *MIR146A* in Treg induces a loss in immune homeostasis characterized by IFN-γ mediated lesions of multiple organs [78]. Hence, miR-146a-5p is considered a tumor suppressor in B-cell malignancies, esophageal cancer, glioblastoma, myeloid malignancies, NK/T-cell lymphoma, ovarian cancer, pancreatic cancer, penile cancer, prostate cancer, and renal cancer and a oncomiR in ALL, AML, bladder cancer, cervical cancer, endometrial cancer, melanoma, multiple myeloma, osteosarcoma, and T-cell leukemia and lymphoma. Furthermore, in several cancers like HCC, breast cancer, gastric cancer, CRC, NSCLC, oral cancer, and thyroid cancer, miR-146a-5p was reported both as a tumor suppressor and an oncomiR [79]. Another such role, is played by the let-7 family miRNAs, which are generally regarded to be tumor suppressor miRNAs in multiple cancer types, playing important roles in inhibiting EMT, invasion, metastasis, and self-renewal [80]. However, in some situations, especially if overexpressed in the **tumor microenvironment** (TME), the let-7 family miRNAs play oncogenic roles. For example, in tumor-associated macrophages let-7d-5p promotes a protumorigenic M2 phenotype characterized by increased tumor burden [81]. These data reveal the heterogeneity of cancer and the multifaceted role miRNAs can have in malignant

pathophysiology, where the miRNA can play different roles in different cancers, stages of carcinogenesis, subtypes of cancer, and in the TME. Future studies are necessary to decipher these context dependent roles and the mechanisms that regulate them.

4. Non-canonical miRNAs functions in cancer

Unconventional localizations and novel interactions with DNA, non-mRNA transcripts, and proteins have provided evidence towards miRNAs being implicated in the regulations of gene expression outside the classic mechanism of target downregulation via recruitment of the RISC to mRNAs. Several databases have been generated to study these non-canonical functions (**Table 1**).

4.1. MiRNAs directly regulating transcription in the nucleus

It is well-known that specific hexanucleotide terminal motifs in miRNAs can regulate the relocation of distinctive miRNAs back into the nucleus [82]. Additionally, AGO1 and AGO2 proteins can enter the nucleus via Importin 8 [83]. These observations opened new avenues to miRNA research that try to understand the nuclear role of miRNAs. It was initially shown that in the nucleus, miR-589-5p forms a complex with AGO2 and GW182. The complex binds directly to the promotor RNA of cyclooxygenase-2 (COX2), thereby activating its transcription [83]. In cancer, several such examples have been discovered [84]. MiR-211-5p is one such example: it is activated by the endoplasmic reticulum stress response and imported into the nucleus where it directly binds the proximal promotor of the pro-apoptotic transcription factor C/EBP homologous protein (CHOP) [85]. At the promotor site, miR-211-5p increases histone methylation, inhibiting the transcription of CHOP and hence, delaying apoptosis (**Figure 2**). In mammary tumors and lymphomas, miR-211-5p is overexpressed and anti-correlates with CHOP, indirectly blocking apoptosis and providing a pro-survival signal [85].

4.2. miRNAs interacting with non-AGO proteins

MiRNAs show a cell type AGO-specific loading pattern with both spatial and temporal variations [86]. However, miRNAs were reported to interact also with non-AGO proteins and this type of

interaction were shown to play an important role in tumorigenesis. Very interesting it was reported that miR-328-3p is downregulated during the blast crisis of chronic myelogenous leukemia [87]. The study revealed that miR-328-3p plays an important role in inducing differentiation of blasts. Mechanistically, miR-328-3p directly binds the translational regulator poly(rC)-binding protein hnRNP E2 desupressing *CEBPA* mRNA, a hematopoietic transcription factor that induces differentiation. The entire mechanism is possible because miR-328-3p harbors a C-rich sequence very similar to the *CEBPA* mRNA spacer region that is recognized by hnRNP E2 in order to induce its inhibition (**Figure 2**) [87].

4.3. MiRNAs activating Toll-like receptors

One unconventional role of miRNAs not fully explored is their ability to directly activate Toll-like receptors (TLRs). This interaction was initially discovered simultaneously in cancer and in neurodegenerative pathology [88, 89]. Lehmann *et al.* observed that let-7b-5p has a GUUGUGU motif similar to sRNA40 derived from HIV, a known activator of TLR7, hence, hypothesizing that this miRNA could activate TLRs. Indeed, microglia and macrophages incubated with let-7b-5p are activated via TLR7, releasing tumor necrosis factor-alpha (TNF-alpha) which induce neurodegeneration. More remarkable was the fact that let-7b-5p was overexpressed in cerebrospinal fluid of Alzheimer's disease patients, partially explaining the spread of central nervous system damage in this disease [89]. Almost simultaneously, Fabbri *et al*., discovered that tumor cells can secrete **exosomes** containing miR-21-5p and miR-29a-3p that bind murine TLR7 and human TLR8 on immune cells and activate a pro-tumorigenic inflammatory response. At a phenotypical level, activation of TLRs induces metastatic spread and tumor growth. MiR-21-5p and miR-29a-3p also have GU rich sequences, GUUG and GGUU, respectively [88]. These observations reveal the importance of the miRNA structure and nucleotide sequence, even outside the seed region (**Figure 2**). Hence, in-depth analysis of miRNA structures is needed in order to unravel unconventional miRNA functions. The clinical value of TLR interaction in cancer patients was revealed in a subsequent study [90]. Here, miR-29a-3p was upregulated in patients with acute Graft Versus Host Disease (aGVHD), and the hyperinflammatory reaction observed in this patient group was partially explained by the miRNA interacting with TLRs on dendritic cells. Moreover, treating a mouse model of aGVHD with locked nucleic acid anti-miR-29a-3p improved the outcome of the mice [90]. Additionally, it was observed that viral miRNAs (**BOX 3**), up-regulated in plasma of patients with sepsis or

surgical trauma, have the capacity to bind TLRs and induce an IL-1b, IL-6 and IL-10 mediated inflammatory reaction. Kaposi's sarcoma-associated herpesvirus (KSHV) miRNAs, kshv-miR-K12-10b and kshv-miR-K12-12-5p can activate TLR8 playing a functional role in the pathophysiology of sepsis [91]. These findings are of great interest especially in the implementation of miRNA mimetics therapy. It is possible that the hyperinflammatory reaction observed in clinical trials after administration of miRNA mimetics may be induced by the activation of TLRs, and strategies to hinder this interaction are highly necessary to implement miRNA therapy [6].

4.4. Pri-miRNAs coding for peptides

Initially discovered in plants, some pri-miRNAs encode small peptides, termed miRNA encoded peptides (miPEPs). *Pri-miR-171b* in *Medicago truncatula* and *pri-miR-165a* in *Arabidopsis thaliana* encode short functional peptides, miPEP-171b – 9 amino acids (aa), and miPEP-165a – 18 aa. The function of these peptides, after reentering the nucleus, is to up-regulate the transcription of the corresponding pri-miRNA in a feed-forward loop and induce the accumulation of their mature forms [92]. In cancer, it was discovered that pri-miRNAs can be translated into peptides/proteins: the pri-miRNAs transcribed from *MIR200A* and *MIR200B* encode miPEP-200a (187 aa long) and miPEP-200b (54 aa long), respectively (**Figure 2**). These miPEPs play an anti-oncogenic role by inhibiting the migratory potential of prostate cancer cells by downregulating vimentin, a key molecule of EMT [93]. Peptides are currently more studied in long ncRNAs (lncRNAs) and circular RNAs (circRNAs), and two ncRNA peptide databases were recently published [94, 95]. As pri-miRNA transcripts are in fact lncRNAs, we therefore hypothesize that future studies will detect more examples of miPEP's abnormal and pathogenic role in cancer.

4.5. Other potential non-canonical functions of miRNAs in cancer

It has been shown that miRNAs can target nuclear ncRNAs and inhibit their function. One example is, miR-709-3p, that localize intranuclear where it binds to the *pri-miR-15a* and *pri-miR-16-1* inhibiting their maturation [96]. MiR-15a and miR-16-1 are well characterized tumor suppressor miRNAs with important role in inducing apoptosis [97]. Indeed, miR-709-3p, via this

inhibitory loop, blocks cells from inducing apoptosis. Therefore, although this discovery was made in mice, we speculate that the miR-709-3p nuclear function could play a role in tumorigenesis [96]. Similarly, it was shown that miR-9-5p together with AGO2 can bind *MALAT1* in the nucleus and downregulate its expression [98]. This discovery was made in Hodgkin lymphoma and glioblastoma cell lines, and *MALAT1* is a well-known cancer associated lncRNA [99].

It has been shown that miRNAs can up-regulate transcription, not just inhibit it: in a cell-cycle dependent manner, miR-369-3p can inhibit or activate translation [100]. MiR-369-3p together with AGO2 and fragile X mental retardation-related protein 1 associate with AU-rich elements in the 3'UTR of *TNF-alpha* mRNA and activate its translation during cell-cycle arrest and inhibit it during the proliferative phase [100]. Taking into account the role played by TNF-alpha in cancer, it is possible that this mechanism is also present in cancer cells. Additionally, it was shown that miR-10a-5p binds to the 5'UTR of multiple ribosomal proteins and activates their translation [101]. Overexpression of miR-10a-5p can activate oncogenic transformation, and activated mouse fibroblasts showed increased colony formation and anchorage-independent growth after transfecting the cells with miR10a-5p. This mechanism is probably mediated by the capacity of miR-10a-5p to activate translation, but further analyses are required [101].

4.6 Interplay between canonical and non-canonical functions

It is important to mention that for most studies where a non-canonical function was identified, the canonical functions was not inhibited or corrected for. However, some non-canonical functions were found to be working in synergy with the canonical one. One such example is the already mentioned miR-328-3p that plays an important role in the differentiation of leukemic blasts. This miRNA exerts its function both in a canonical and non-canonical way. Canonical, by binding the 3'UTR of and post-transcriptionally inhibiting the mRNA of the survival factor *PIM1* and non-canonical, by interacting with the non-AGO protein hnRNP E2 [87]. Therefore, a synergism can exist between the canonical and non-canonical functions of a miRNA. Another mentioned example is that of *pri-miR-171b* and *pri-miR-165a* that are translated into peptides. The only known function of these peptides is to activate the transcription of their host pri-miRNAs, inducing the accumulation of the mature forms of miR-171b and miR-165a that exercise their canonical function in cytoplasm [92]. Hence, the non-canonical function can potentiate in a feed-forward lops the canonical miRNA function. We believe that similar

interactions between the two types of functions exist and need to be further researched. To our knowledge, there is no specific method to exclude canonical effects of miRNAs with non-canonical function. In most cases, researchers used knock in, knock out, and rescue experiments of the miRNAs and their downstream non-canonical interactors to prove their atypical functions. Moreover, a simple trick to prove the non-canonical function in cases where this function is AGO independent is to inhibit AGO (i.e. pri-miRNAs coding for peptides, miRNAs interacting with non-Ago proteins, and miRNAs activating Toll-like receptors). However, such studies will cause a general downregulation of all miRNAs, with potential large changes in the cell's transcriptome and proteome.

5. Concluding Remarks

Although almost 20 years have passed since the discovery of miRNAs being implicated in carcinogenesis [1], this captivating and many-sided class of transcripts have not found their way into clinical practice (**BOX 4**). The plethora of recent discoveries highlighted in this review could make this translation possible.

Regarding miRNAs as biomarkers, firstly, now we know that one miRNA is not enough for developing a diagnostic tool, and miRNA networks are necessary for creating new miRNA based diagnostic approaches with a desired diagnostic power [102]. We also consider that the miRNome can be used for subclassifying malignant entities and for separating tumors with similar phenotype and even discover new malignant sub-entities. Secondly, we consider that by adding information about the mechanisms used by miRNAs to travel in bodily fluids (i.e. exosomes, lipids, and proteins) we can increase the specificity of the diagnostic tools (**Table 1**). Thirdly, analyzing miRNAs that are chemically modified or edited could provide additional diagnostic power for employing these molecules as biomarkers. Fourthly, adding **isomiRs**, that are expressed in a tissue and disease specific manner, in diagnostic algorithms could provide the necessary diagnostic specificity. IsomiRs can be generated by editing events happening after maturation, including modification of the 3' end of the miRNA by nucleotide transferase (adenylation or uridylation) and 3′-exonuclease processes [7, 103]. IsomiRs can also be generated by events before maturation. Conformation changes in the pri-miRNA hairpin structure or imprecise cleavage of pri- and pre-miRNAs by DROSHA and DICER1 can cause formation of isomiRs. Very interesting, specific isomiRs formation has been found to be tumor

specific and can separate between tumor types much better that current expression-based classifiers [104]. This observation can represent a key step towards miRNA biomarker development. Moreover, the breakthrough may come from new technologies used for improving cancer diagnostics, like the analysis of miRNA expression in circulating tumor cells or at the single cell level.

Regarding miRNA therapy [6, 105, 106], firstly, it became clear that miRNA mimetics similar to viral RNA particles can induce an uncontrolled inflammatory response via activating TLRs, and methods need to be developed to avoid this interaction. The recently approved mRNA vaccines for COVID-19, uses modified RNA molecules in order to prevent adverse immune response via activation of TLRs [107]. The vaccines have proven to be highly effective, and now major effort is being put forward to develop mRNA vaccines against other diseases, including cancer [107]. Secondly, miRNA therapy can be used as an adjuvant that potentiates the effect of standard therapies like chemo-, radio-, or immunotherapy [108]. Thirdly, a successful miRNA therapy needs an ingenious delivery mechanism that permits the transfer of miRNAs only into cancer cells. One way to avoid several obstacles is to target miRNAs (as well as other ncRNAs) that are specifically present in malignant cells, but are not expressed in the normal cells from the same tissue type as the tumor. In this way, the miRNA targeting is more specific, less toxic, and easier to quantify, as normal cells have no or too little amount of the specific ncRNA.

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Declaration of interests

Dr. Calin is the scientific founder of Ithax Pharmaceuticals. The other authors declare no conflict of interest.

FIGURE 1: Mechanisms of miRNA dysregulation **in cancer.** MiRNAs can be dysregulated in cancer via multiple mechanisms. At the DNA level, genomic loss or amplifications of miRNA genes or single nucleotide mutations with consequences for miRNA processing or target recognition can occur during tumorigenesis. Further, epigenetic modifications can cause dysregulated expression. Dysregulated expression could also be a consequence of loss or overexpression of key miRNA biogenesis proteins and membrane transporters or by chemical modification and editing processes of the primary (pri), precursor (pre), or mature miRNA. Finally, an indirect mechanism that can control the expression of miRNAs in different bodily comportments (intracellular and extracellular) is the miRNA trafficking.

FIGURE 2: Non-canonical miRNAs functions in cancer (Key Figure). Several functions, outside the traditional 3' UTR target recognition of mRNAs, has been described for miRNAs and has been implicated in tumorigenesis. These include direct gene regulation in the nucleus via promoter interactions, activation of endosomal Toll-like receptors (TLRs), regulation of gene expression after assembly with non-AGO proteins, and miRNA transcripts coding for peptides. Additionally, miRNA sponges and viral miRNAs have been implicated in tumorigenesis, but are not classifies as unconventional functions.

TABLE 1: Databases for studying miRNA non-canonical functions.

Unconventional miRNA function	Database	Description of Database		Ref.
ncRNAs coding for peptides	FuncPEP	A manually-curated database that contains all functional peptides (< 100 aa) that are coded by ncRNAs, including lncRNA, circRNAs, tRNAs and miRNAs.	https://bioinformatics.mdanderson.org/Supplements/FuncPEP/	[94]
	ncEP	A manually-curated database of ncRNAs that encode for experimentally validated peptides.	http://www.jianglab.cn/ncEP/	[95]
miRNA interacting with other proteins	SimiRa	A database of miRNAs and RNA binding proteins with shared function, including pathways and GO terms.	http://vsicb-simira.helmholtz-muenchen.de.	[109]
	DoRiNA 2.0	A database of miRNA and RNA binding proteins with shared post-transcriptional function.	http://dorina.mdc-berlin.de	[110]
miRNA sponging	miRSponge	A manually curated database that contains only experimentally validated miRNAs and the ncRNA molecules that sponge them.	http://www.bio-bigdata.net/miRSponge	[111]
	miRTissue $_{ce}$	A database containing sponge type interactions between miRNAs and other ncRNAs in different cancer tissue types.	http://tblab.pa.icar.cnr.it/mirtissue.html	[112]
	miRNAsong	A database that generates *in silico* potential miRNA sponges.	http://www.med.muni.cz/histology/miRNAsong/	[113]
viral miRNAs	VIRmiRNA	A database containing experimentally validated miRNAs and their targets and anti-viral miRNAs, endogenous miRNAs that potentially target viral genomes.	http://crdd.osdd.net/servers/virmirna	[114]
	Xeno-miRNet	A comprehensive database and analytics platform to explore xeno-miRNAs and their potential targets.	http://xeno.mirnet.ca	[115]
miRNA regulating transcription	MicroPIR2	Predicts miRNA – promotor interaction in human and mouse.	http://www4a.biotec.or.th/micropir2	[116]
	miRactDB	A database that analysis direct, and indirect miRNA gene interactions and the consequences of the miRNA-gene relation in multiple tissues, in normal and cancer. The database is focused mainly on miRNA gene coding sequence and promotor interaction.	https://ccsm.uth.edu/miRactDB	[117]
Other atypical miRNA functions	STarMirDB	A database of predicted miRNA binding sites based on CLIP studies. The database includes both seed and seedless binding sites, and sites located both in the canonical 3' UTR, and in the atypical sites in CDS and 5' UTR of mRNAs.	http://sfold.wadsworth.org/starmirDB.php	[118]
	miR*SNP*	A collection of SNPs in miRNA-mRNA sites and predictions regarding the miRNA-mRNA interactions for the different alleles.	http://cmbi.bjmu.edu.cn/mirsnp	[119]
	PolymiRTS 3.0	A database corroborating miRNA expression, seed region DNA variants and the resulting phenotype.	http://compbio.uthsc.edu/miRSNP	[120]
	EVmiRNA	A database of miRNAs enclosed in extracellular vesicles based on data from 462 RNA sequencing samples form 17 sources and diseases.	http://bioinfo.life.hust.edu.cn/EVmiRNA	[121]
	miRandola 2017	A manually curated database of miRNAs and other ncRNAs role as potential biomarkers, the database contains data obtained from multiple organisms, diseases, sample types, ncRNA drug interactions, and extracellular transport mechanism.	http://mirandola.iit.cnr.it/	[122]

Text box 1: Circulating miRNAs: from biomarkers to hormones

A highly important discovery regarding the role of miRNAs in cancer was the observation that miRNAs were identified in bodily fluids and are dysregulated in cancer patients versus normal controls. In 2008 the group of Muneesh Tewari discovered the presence of miR-141-3p in plasma [123]. MiR-141-3p was upregulated in prostate cancer patients versus healthy controls, being a potential biomarker for this patient group with an area under the curve of 0.907 [123]. In the following years it was observed that miRNAs were present and dysregulated also in other bodily fluids, like bile of biliary tract cancer patients [124], urine of bladder cancer patients [125], stool of colorectal cancer and ulcerative colitis [126], and saliva of patients with oral cancer [127]. These observations led to the assumption that miRNAs are the next generation of non-invasive biomarkers [5]. Unfortunately, up to date none of these findings has been successfully translated into clinical practice, mainly due of the low specificity of the dysregulated miRNAs – often the same miRNAs are dysregulated in multiple types of cancers and non-malignant diseases. Further, this transition has also been challenged due to the lack of reproducibly in miRNA isolation and detection. This might be overcome by analyzing specific subtypes of circulating miRNAs: precise fluid localizations like exosomes, specific isomiRs species, or specific chemically modified miRNAs.

In parallel, researchers tried to understand how miRNAs travel in bodily fluids and to establish their biological function. It was observed that miRNAs can be transferred between cells via exosomes [128], or bound to proteins like AGO2 [129] and lipids like high-density lipoprotein (HDL) [130]. Nowadays, we perceive miRNAs as the smallest type of hormones acting in an autocrine, paracrine, and endocrine manner. In this regard, we have witnessed some very interesting discoveries explaining the interplay between tumor cells and different components of the TME. For example, we have recently shown that loss of *TP53* in head and neck cancer induces a switch in peritumoral nerve fibers from sensory type to adrenergic type [131] via exosome signaling from tumor cells containing the oncomiRs miR-21-5p and miR-324-5p and lacking the tumor suppressor miR-34a-5p. Adrenergic nerve fibers are well-known to promote tumorigenicity, hence, a feed-forward loop is created that ensures tumor growth. Moreover, the miRNA transfer is not unidirectional from tumor cells to TME, but also the other way around. For example, it was shown that polymorphonuclear leukocytes release exosomes containing miR-223-3p. These exosomes are engulfed by cancer cells, and intracellular miR-223-3p can induce a transitory EMT phenotype by inhibiting FOXO1 [132]. Transfer of circulating, tumor

derived miRNAs was shown to regulate other cancer-TME crosstalk mechanisms like formation of the metastatic niche [133, 134] and regulation of the immune response [135].

Additionally, extracellular miRNA trafficking can be perceived as another potential mechanism that controls the number of intracellular miRNAs by regulating the internalization and externalization of miRNAs from and into the extracellular milieu [136]. This is not *per se* a mechanism that changes the overall expression of miRNA, but one that can be perceived as an indirect cause of the dysregulation of miRNAs in tissues and/or bodily fluids of cancer patients (**Figure 1**).

Text box 2: miRNA sponges

MiRNA sponging refers to the capacity of miRNAs to bind ncRNAs that sequester miRNAs and prevents target recognition. This mechanism was initially discovered in plants, where in *Arabidopsis thaliana* the ncRNA *IPS1* binds and sequesters ath-miR-399-3p resulting in the overexpression of the mRNA *PHO2,* the canonical target of ath-miR-399-3p, modifying the phosphate metabolism [137]. MiRNA sponges have been constructed and inserted in human cells showing that artificial sponges can inhibit miRNA functions [138], and this method is now considered a miRNA inhibition method. Subsequently, the role of miRNA sponging was described also in cancer. Here, it was showed that a pseudogene of PTEN, *PTENP1*, binds and sequesters several miRNAs that inhibit the tumor suppressor PTEN (**Figure 2**). These data proved that a pseudogene can have a miRNA mediated non-coding function that indirectly suppresses tumor growth [139]. It was further proved with various levels of details that almost every well characterized lncRNA and circRNA functions as a miRNA sponge [140]. This has led to a network theory interpretation, where coding and ncRNA nodes are linked via edges that represent direct interaction [102]. Most probably, this mRNA-miRNA-ncRNA crosstalk is possible only in specific sub-cellular compartments where unphysiologically high levels of miRNA response elements (MRE) are reached [141, 142], and intracellular transport mechanisms of miRNAs could play a crucial role [143]. We believe that in order to further analyze in a critical manner the role of miRNA sponging in cancer, a laborious methodology is necessary that must employ multiple direct interaction tools (RNA immunoprecipitation, protein pull-down, luciferase assay), co-localization studies (florescence in situ hybridization), knock in and knock out studies using genome editing, and transcription kinetics studies using new RNA-seq data combined with mathematical models [142, 144].

miRNA can bind not only to human sequences but also to viral RNAs. miRNAs can mitigate the pathogenesis of COVID-19 disease via binding to the SARS-CoV-2 genome and inhibit its post-transcriptional expression [145-150]. MiRNAs such as miR-21-3p, miR-195-5p, miR-16-5p, miR-3065-5p, miR-424-5p and miR-421 potentially regulate all human coronaviruses through direct binding to their viral genome [150].

Text box 3: Xeno-miRNAs: from miRNA mimicry to cancer biomarkers

It was discovered that viruses' express miRNAs, termed xeno-miRNAs, that play a role in sustaining different phases during viral infection of human cells. Xeno-miRNAs were initially discovered in Epstein-Barr virus (EBV) [151] and subsequently in Kaposi's sarcoma-associated herpesvirus (KSHV) [152], linking them with cancer biology. Indeed, quickly after their discovery it was noted that EBV BART miRNAs are expressed in EBV associated gastric cancer, suggesting that these viral miRNAs play a tumorigenic role [153]. Very interesting some of the viral miRNAs are orthologous of endogenous (cellular) miRNAs, like miR-K12-11 and miR-155-5p, resulting in target mimicry, that has a pathogenic role in B-cell lymphoproliferative disorders (**Figure 2**) [154]. Furthermore, xeno-miRNAs are transferred from EBV infected cells via exosomes into other cell types where they exert gene repression [155]. The xeno-miRNAs are also expressed and circulate in plasma of patients with CLL. Ebv-miR-BHRF1-1 is overexpressed in CLL patient's plasma and associates with several established markers of unfavorable prognosis, like advanced RAI stage (one of the most widely used CLL staging systems), and beta-2-microglobulin levels, and their levels can be used to predict the survival of these patients. High levels of ebv-miR-BHRF1-1 correlate with miR-155-5p and with a downregulation of TP53 [156]. In glioblastoma, several viral miRNAs were found to be overexpressed in plasma: EBV miRNAs - ebv-miR-BART15, ebv-miR-BART2-5p, ebv-miR-BART6-3p, ebv-miR-BART9, ebv-miR-BHRF1-3; human cytomegalovirus miRNAs - hcmv-miR-US5-2, herpes simplex virus 1 miRNAs - hsv1-miR-H1; and a KSHV miRNA - kshv-miR-K12-7, while other two were downregulated: ebv-miR-BART2-3p and hsv1-miR-H4-5p [157]. These results show that xeno-miRNAs have the potential of being attractive cancer biomarkers.

Text box 4: MiRNA therapy: inhibiting the oncomiRs and replacing the tumor suppressor miRNAs

The inhibition of up-regulated oncomiRs is generally termed anti-miRNA therapy, and the restoration of tumor suppressor miRNAs is termed miRNA mimetics therapy [158]. The arsenal of inhibiting miRNAs is vast, and it includes multiple types of antisense nucleotides that directly bind oncomiRs and induce their degradation (i.e. antisense oligonucleotides targeting miRNAs (AMOs), locked nucleic acids anti-miRs (LNA-anti-miRNAs), and antagomirs). An alternative to these are the antisense oligonucleotides that bind the 3'UTRs of mRNA targets of oncomiRs (miRNA masks), circRNAs that bind and sequester miRNAs (artificial miRNA sponges), and molecules that interfere with miRNA biogenesis (small-molecule inhibitors of miRNAs) [6, 158]. All these compounds were tested in cancer research *in vitro* and *in vivo* but were never used in the clinical setting. In non-malignant diseases anti-miRNA therapy was tested in Phase 1 and Phase 2 clinical trials. Here, LNA against miR-122-5p were developed with promising results and only mild side effects for patients with hepatitis C virus [159]. However, long term therapy was associated with resistance due to mutations of the RNA virus [160, 161].

MiRNA mimetics are chemically modified double stranded miRNA-like molecules, that upon delivery into the intracellular milieu are incorporated in the RISC complex and can induce the inhibition of their target mRNAs. The effect of miRNA mimetics therapy was studied also in the clinical setting. The best-known example is that of MRX34, a double strand miR-34a-5p mimic incorporated in liposomal nanoparticles. After promising results *in vivo* [162] the drug was tested in the first miRNA based clinical trial (NCT01829971) in humans with advanced solid cancer. Because of serious adverse events, including four drug-related deaths the study was terminated early. The most serious adverse events were mimicking systemic inflammatory response syndrome (SIRS) [163] making us hypothesize that the mechanism may be mediated by the capacity of miR-34a-5p mimetics to bind TLRs. Nevertheless, three patients showed partial response and 16 showed a clinical stable disease [163]. More ingenious clinical trials fallowed, through incorporation of miR-16-5p mimics in minicells (termed TargomiRs) patients with malignant pleural mesothelioma were IV treated (NCT02369198). Of the 22 patients analyzed, one showed partial response and 15 stable disease [164].

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CLASSICS AND NON-CANONICAL FUNCTIONS OF MIRNAS IN CANCERS

Highlights

- Germline and somatic mutations of miRNAs, their targets, and processing proteins have major implication in cancer initiation and progression.

- Epigenetic regulation and modification of primary, precursor and mature miRNAs transcripts, in addition to miRNA biogenesis proteins, are additional regulatory mechanisms for miRNA transcription, maturation, and target recognition implicated in cancer.

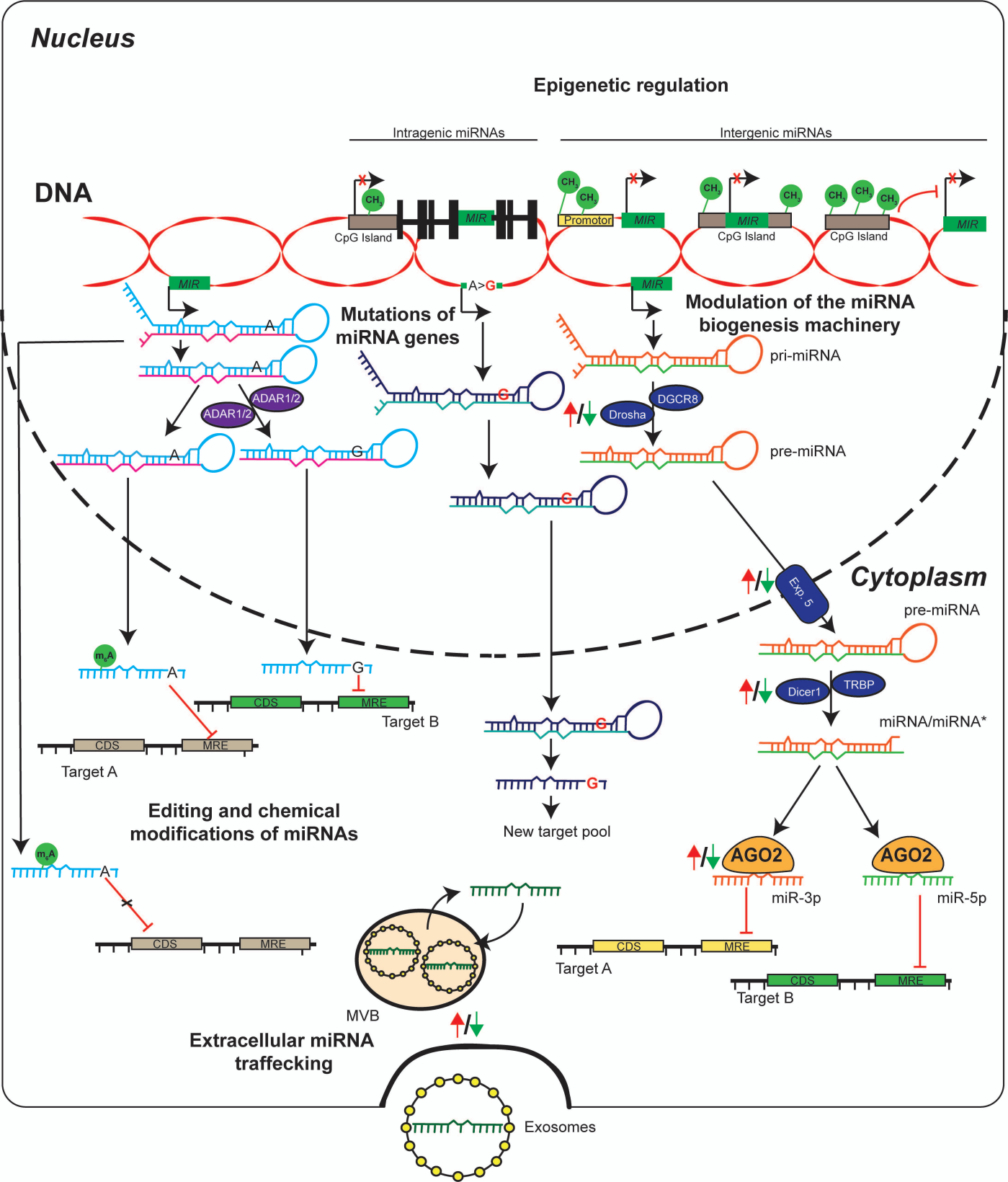
- The number and extent of non-canonical functions of miRNAs are increasing and are associated with cancer.

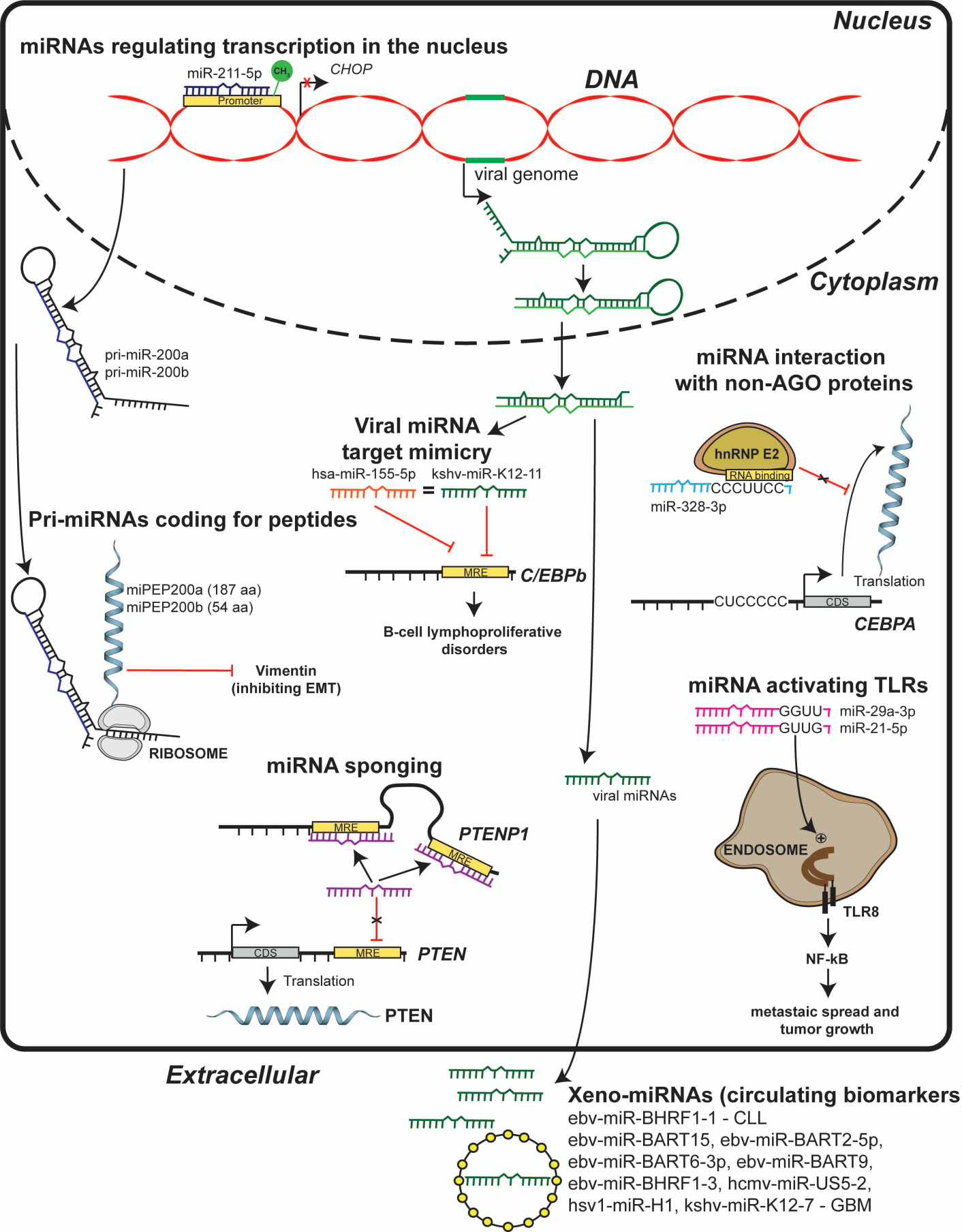
- Viral miRNAs (xeno-miRNAs) have similar sequences to human miRNAs sharing a similar pool of targets (target mimicry) and are secreted into bodily fluids in malignant diseases, and thereby have potential as novel cancer biomarkers.

CLASSICS AND NON-CANONICAL FUNCTIONS OF MIRNAS IN CANCERS

Outstanding questions

- We perceive all the atypical mechanism as non-canonical miRNA functions and important literature supports their role in cancer. *How prominent are these non-canonical functions of miRNAs in cancer cells and how many others will be discovered further?*
- For an accurate genetic diagnosis of cancer risk, both DNA (for SNP alleles or silent mutations identification) and paired RNA (for interactor miRNA profiling) from the same individual germline and tumor must be tested. Only in this way can the actual risk that is conferred by both interactor partners, the dysregulated interactor microRNA and the altered interactor element from the coding sequence of the mRNA, be assessed. *Are we prepared to reorganize the tissue samples biobanks to adjust for the needs to identify at-risk persons where miRNAs (meaning RNAs and not mutated DNAs) are involved in genetic predisposition?*
- The importance of miRNA post-transcriptional modification for its expression, mode of action, and as more specific biomarkers are scantly studied. For example, DNA hydroxymethylation (the addition of a hydroxyl group on 5-methylcytosines of CpG dinucleotides resulting in a 5-hydroxymethylcytosine) activates transcription, and recently DNA hydroxymethylation was reported to regulate miR-365-3p. *How widespread are these miRNAs post-transcriptional modification in cancer cells and in the TME, and what are their functional roles in cancer initiation and development?*
- The effects of phylogeny through ultraconservation or primate-specific occurrence on miRNAs expression and spectrum of targets, has still to be explored. *Which are better suited as biomarkers and for miRNAs therapeutics, the well conserved or the human/primate specific miRNAs?*
- Anti-miRNA therapy uses various categories of small RNA-derived molecules. An additional way to inhibit oncogenic overexpressed miRNAs, in addition to RNA viruses such as SARS-CoV-2, is through small molecules inhibitors. *How efficient are these molecules, and what are their spectrum of toxicities as compared with small RNA drugs?*


Nucleus
Epigenetic regulation
Intragenic miRNAs
Intergenic miRNAs
DNA
CpG Island
MIR
Promotor
A>G
Mutations of miRNA genes
Modulation of the miRNA biogenesis machinery
pri-miRNA
DGCR8
Drosha
pre-miRNA
ADAR1/2
Cytoplasm
Exp. 5
pre-miRNA
Dicer1
TRBP
miRNA/miRNA*
AGO2
miR-3p
miR-5p
CDS
MRE
Target A
Target B
New target pool
Editing and chemical modifications of miRNAs
MVB
Extracellular miRNA traffecking
Exosomes


Nucleus
miRNAs regulating transcription in the nucleus
miR-211-5p
CH3
Promoter
CHOP
DNA
viral genome
Cytoplasm
pri-miR-200a
pri-miR-200b
miRNA interaction
with non-AGO proteins
Viral miRNA
target mimicry
hsa-miR-155-5p
kshv-miR-K12-11
=
hnRNP E2
RNA binding
CCCUUCC
miR-328-3p
Pri-miRNAs coding for peptides
MRE
C/EBPb
B-cell lymphoproliferative
disorders
Translation
CUCCCCC
CDS
CEBPA
miPEP200a (187 aa)
miPEP200b (54 aa)
Vimentin
(inhibiting EMT)
RIBOSOME
miRNA activating TLRs
GGUU
miR-29a-3p
GUUG
miR-21-5p
miRNA sponging
viral miRNAs
MRE
PTENP1
MRE
ENDOSOME
TLR8
CDS
MRE
PTEN
NF-kB
Translation
PTEN
metastaic spread and
tumor growth
Extracellular
Xeno-miRNAs (circulating biomarkers)
ebv-miR-BHRF1-1 - CLL
ebv-miR-BART15, ebv-miR-BART2-5p,
ebv-miR-BART6-3p, ebv-miR-BART9,
ebv-miR-BHRF1-3, hcmv-miR-US5-2,
hsv1-miR-H1, kshv-miR-K12-7 - GBM