MicroRNA history: Discovery, recent applications and next frontiers

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Abstract

Since 1993, when the first small non-coding RNA was identified, our knowledge about microRNAs has been exponentially growing. In this review we intend to focus on the main progresses on this field and to discuss the most important findings under a historical perspective. In addition, we examine microRNAs as disease diagnosis and prognosis markers as well as new therapeutic targets.

Introduction

microRNAs (miRNAs) are small non-coding RNAs (ncRNAs) with approximately 20 nucleotides (nt) length that regulate gene expression posttranscriptionally by binding to 3'untranslated regions (UTR), coding sequences or 5´UTR of target messenger RNAs (mRNAs) and leading to inhibition of translation or mRNA degradation [1,2,3]. It is predicted that miRNAs regulate approximately 30% of the human protein-coding genome [1]. miRNAs control the expression of genes involved in several biologic processes such as apoptosis, proliferation, differentiation and metastasis [1,2,3].

Two decades ago, the existence and importance of miRNAs was completely unknown. Until then, the scientific community was focused on genes that codify for protein. The classical dogma that DNA is transcribed into RNA which then is translated into protein put aside the study

of all the non-protein coding sequences. Only in 1993 the importance of miRNAs started to be revealed [4,5]. This review intends to highlight the pioneer studies that contributed to the brief history of miRNAs (Figure 1).

Discovery of the first miRNA: *lin-4*

Lin-4 was the first miRNA to be discovered, in 1993, by the joint efforts of Victor Ambros's and Gary Ruvkun's laboratories [3,4]. In the nematode *C. elegans*, heterochronic genes control temporal development pattern of all larval stages. One of these genes is *lin-4*, discovered by the isolation of a null mutation that causes a failure in temporal development [6,7]. Animals with lin-4 loss-of-function mutations are missing some adult structures, are unable of laying eggs and reiterate early development programs at inappropriate late larval stages. Lin-4 activity is required for the transition from L1 to L2 stage of larval development [7,8]. In 1987, Fergunsson *et al.*, at Hortitz's lab, found that a suppressor mutation in the gene *lin-14* was able to revert the *null-lin-4* mutation phenotype [9]. In fact, null mutations in *lin-14* gene caused an exactly opposite phenotype of the *null-lin-4* mutations [8,9]. The interesting opposite phenotype between defects in *lin-4* and *lin-14* genes indicated that lin-4 could negatively regulate lin-14 [8]. In 1989, Ambros worked with Ruvkun, at Horvitz's lab, to clone lin-14 gene [8,10]. At this time, the two colleagues followed two independent careers: Ambros focus his research on *lin-4* gene and Ruvkun on the study *lin-14* gene.

Ambros, together with Rosalind Lee and Rhonda Feinbaum, found that a 700bp fragment could contain *lin-4* gene but could not find the conventional start and stop codons. Even so, they introduced mutations in the putative ORFs but lin-4 function remained unchanged. Therefore, Ambros concluded that *lin-4* did not encode a protein [4,8]. In addition, they found two very small lin-4 transcripts with only 61 and 22 nt length [4]. On the other hand, Ruvkun and his colleagues Bruce Wightman and IIho Ha, found that *lin-14* was downregulated at a posttranscriptional level and that *lin-14* 3´UTR region was sufficient for the temporal regulation [5]. The two groups shared their unpublished data and in June 1992 Ambros and Ruvnkun independently came to the same conclusion: *lin-4* transcripts were complementary to a repeated sequence in the 3´UTR of *lin-14* gene [4,5,8]. In December 1993, in the same issue of Cell, Ambros and Ruvkun independently reported that the small and non-protein coding transcript *lin-*

4 regulates *lin-14* through its 3' UTR region [4,5,8]. A new unexpected cellular regulatory mechanism involving a non-protein coding transcript had been found!

Discovery of a second microRNA: Let-7

Likewise lin-4, let-7 is a heterochronic gene of *C. elegans* and was the second miRNA to be discovered, in 2000, seven years after the finding of the first miRNA. Reinhart, Slack *et al.* at Ruvkun's lab reported that *let-7* was a 21 nt RNA controlling L4-to-adult transition of larval development [11]. Loss of let-7 activity causes reappearance of larval cell fates during adult stage of development, while increased let-7 activity causes precocious expression of adult fates [11]. Remarkably, the authors found that the retarded let-7 phenotype could partially suppress *lin-41* (a *let-7* target) loss-of-function mutations [11]. In fact, *let-7* is complementary to two closely spaced sites in *lin-41* 3´UTR. Deletion of the *lin-41* 3'UTR region and *let-7* mutations abolishes *lin-41* downregulation, showing that both partners are necessary for this mechanism [11,12,13].

Let-7 controls late temporal transitions during development across animal phylogeny. Unlike *lin-4*, *let-7* sequence is conserved across species from flies to humans [14], which had a major impact on the study of this specific miRNA in other organisms [15,16]. *let-7* RNA was detected in vertebrate, ascidian, hemichordate, mollusc, annelid and arthropod, but not in RNAs from plant and unicellular organisms [14,16]. In humans, it was detected at different expression levels in the majority of the tissues, including brain, heart, kidney, liver, lung, trachea, colon, small intestine, spleen, stomach and thymus [14]. *Let-7* miRNA family refers to miRNAs that share complete sequence identity with *let-7* at the 5' ends, termed seed regions, and therefore can regulate the same targets [15]. Let-7 family within humans comprises 12 miRNAs. Some members of let-7 family identified and functionally analysed in *C. elegans* were mir-48, mir-84, and mir-241 [17-19]. These miRNAs act redundantly to control the L2-to-L3 transition, by repressing *hbl-1* [19]. Nowadays, functional cooperation among miRNA family members continues to be studied [20].

The discovery that let-7 is conserved across species triggered a revolution in the research of a new class of small ncRNAs, called miRNAs. Presently, thousands of miRNAs had been identified in humans and other species, and miRNA online sequences repositories, such as the

miRbase database, are available [21,22,23]. Also, current tools and software developed for miRNA target prediction facilitate studies of miRNAs functional network [24,25].

miRNA biogenesis pathway and miRNAs function

miRNAs are encoded in the genome as long primary transcripts (named pri-miRNAs) that contain a cap structure at the 5' end and are poly-adenylated at the 3' end. Pri-miRNAs are processed by the cellular RNaseII enconucleaseIII Drosha, together with DGCR8/Pasha proteins, into a structure of 60-110 nt, called precursor-miRNA (pre-miRNA), which is then exported from the nucleus to the cytoplasm by an Exportin-5 dependent mechanism. In the cytoplasm, the pre-miRNA is cleft by the RNaseIII enzyme Dicer-1, together with TRBP/PACT proteins, producing a short imperfect double-stranded miRNA duplex. This duplex is then unwound by an helicase into a mature miRNA with approximately 20 nt length, which is then incorporate in a multicomponent complex constituted by Argonaute family protein members known as RISC [26].

In a historical perspective, the knowlegment acquired from RNAi was determinant for understanding miRNAs processment and activity. In 2000, Zamore *et al.* studied RNAi process and described that double-stranded RNA fragments of 21-23 nucleotides were targeting the mRNA cleavage [27]. The functional unit of RNAi was therefore the same size as miRNAs. In 2001, two reports were crucial for elucidation of the miRNA biogenesis mechanism, as both reports suggest an involvement of RNAi pathway components in miRNAs maturation [28,29]. Grishok and colleagues showed that a homolog of *Drosophila* Dicer (*dcr-1*), and two homologs of rde-1 (*alg-1* and *alg-2*) were essential for lin-4 and let-7 activity. Inactivation of these genes caused phenotypes similar to *lin-4* and *let-7* mutations [28]. Simultaneously, Hutvagner *et al.* described that let-7 pre-miRNA is cleaved by Dicer. In fact, when cells were transfected with siRNA duplex corresponding to human Dicer enzyme, pre-let-7 accumulated into the cells [29]. Taken together, these two papers corroborate the intersection between RNAi and miRNA pathway and opened the door for understanding the formation of mature miRNAs.

In the miRNAs biogenesis pathway, Drosha and Dicer are spatially separated, being localized in the nucleus and in the cytoplasm, respectively. In 2004, Lund *et al.* reported that Exportin-5 was the key player mediating the efficient nuclear export of the short miRNA precursors [30]. However some miRNAs such as miR-29b are predominantly located in the

nucleus [31]. In 2007, Hwang *et al.* found that some miRNAs contain additional sequences elements that control their subcellular localization. In fact, miR-29b possesses a hexanucleotide terminal motif that directs its import into the nucleus. The authors show that, despite their small size, miRNAs can contain additional cis-acting regulatory motifs that might influence their posttranscriptional behavior and concluded that miRNAs with common 5' ends, predicted to regulate the same targets, might have distinct functions [31].

miRNAs' main function is to inhibit protein synthesis of protein-coding genes, either by inhibition of translation or mRNA degradation. However, the relative contribution of each mechanism to repression was unknown until recently. In an elegant study, Guo *et al.* used ribosome profiling to measure the overall effects on protein production and simultaneously measured effects on mRNA levels and concluded that inhibition of translation (no changes in mRNA levels of miRNA targets) had a modest influence on repressing protein levels whereas mRNA destabilization was the predominant miRNAs mechanism of action to decrease their targets levels [32].

Besides mRNAs repression, miRNAs have been also reported to activate translation of targeted mRNAs [33,34]. Vasudevan *et al.* were the first to clearly demonstrate that, in some instances, miRNAs can work as translational activators. Under serum starvation conditions, TNFα AU-rich elements recruited miR369-3 to mediate translation upregulation. However, in synchronized proliferating cells it caused translation repression. In addition, on cell cycle arrest, let-7 and the synthetic miRNA-cxcr4 induced translation whereas they repress translation in proliferating cells. Therefore, miRNAs can switch between translation repression and activation in coordination with cell cycle [34]. In 2008, Place *et al.* provided new evidence that miRNAs can induce gene expression and were the first to show that miRNAs can target genes promoters. The authors showed that miR-373 targets the promoter of E-cadherin and CSDC2 and induced their expression [35].

For a long time, studies on miRNA-target interaction were confined to 3'UTR of mRNAs, probably because the first studies on miRNAs focused on this region. In 2007, Lytle *et al.* was the first to suggest that miRNAs could associate to any position of target mRNAs and demonstrated that mRNAs targets were efficiently repressed by miRNA-binding sites in 5'UTR [36]. In 2008, Tay *et al.* reported that binding sites in CDS are abundant and experimentally

show that mouse *Nanog*, *Oct4* and *Sox2* genes have miRNA-binding sites in their CDS. MiRNAs targeting these genes modulate embryonic stem cell differentiation [37].

In 2010, Eiring *et al.* reported a remarkably finding for our understanding on how miRNAs function. The authors found that, in addition to miRNAs gene silencing activity thought base pairing with mRNA targets, miRNAs also have decoy activity that interferes with the function of regulatory proteins [38]. Particularly, miR-328 binds to hnRNP E2 independently of the miRNA's seed region and prevents its interaction with *CEBPA* mRNA [38]. In conclusion, the authors introduced the new concept that miRNAs can work as molecular decoys for RNA-binding proteins [38].

microRNAs in disease: an historical perspective

microRNAs in Cancer

The first report suggesting a role of miRNAs in cancer was published in 2002 [38]. *MiR-15* and *miR-16* were found to be located at chromosome 13q14, a region frequently deleted in chronic lymphocytic leukemia (CLL). Calin *et al.* discovered that either genes are deleted or downregulated in over 60% of B-cell human CLL, indicating that these genes behave as tumor suppressors in CLL [39]. Consequently, the same group identified that a significant percentage of miRNAs is located at fragile sites and in regions altered in cancers, including regions of amplification or loss of heterozygosity or breakpoints, suggesting that miRNAs as a new class of genes had a relevant role of in human cancer pathogenesis [40].

Oligonucleotide miRNA microarrays and, more recently, deep sequencing (next generation sequencing) has permitted the analysis of the entire known miRNAome. In addition, other methods such as bead-flow cytometry, quantitative real-time polymerase chain reaction and high-throughput array-based Klenow enzyme assay, have been used to assess miRNA expression in tumors and other diseases. To date, altered miRNA expression had been reported in almost all types of cancer [41].

In 2005, the first reports addressing the biological function of miRNAs in cancer were published. MicroRNAs can act as oncogenes (oncomirs) or tumor suppressors and are involved in a variety of pathways deregulated in cancer [42]. In March 2005, Johnson *et al*. reported the first miR-target interaction with relevance to cancer. The authors demonstrate that in *C. elegans* let-7 targets let-60, encoding the *C. elegans* ortholog of human oncogene RAS. In addition, they

show that human RAS expression is regulated by let-7 in cell culture. Accordingly, let-7 expression is decreased in lung cancer comparing to normal tissue and it correlates with the increased RAS proteins levels detected in lung tumor samples [43]. Cimmino *et al.* reported that miR-15 and miR-16, the first two miRNAs associated to cancer, played a role in apoptosis regulation by targeting the anti-apoptotic BCL2 mRNA [44]. Also in 2005, He *et al.* studied for the first time the contribution of miRNAs for tumor development in vivo. By overexpressing miR-17-22 cluster, which is upregulated in human lymphoma, the authors were able to accelerate lymphomagenesis in a mouse B-cell lymphoma model carrying c-myc oncogene [45]. Furthermore, O'Donnell *et al.* reported for the first time that a transcription factor, specifically MYC, modulated the expression of the same cluster of miRNAs and consequently the E2F1 expression [46]. From 2005 until present, hundreds of scientific communications have been reporting on the role of miRNAs in tumors, as well as the regulation of miRNAs by other transcription factors such as TP53 (for a detailed review see [47]). In 2010 Medina *et al.*, reported the results on mice that conditionally overexpressed miR-21 and this clearly demonstrate that overexpression of a single miRNA, specifically miR-21, was sufficient to cause tumors development. In addition, the authors proved that tumor volume and survival were dependent on miR-21 overexpression and that tumor regresses when miR-21 was inactivated, proving for a first time an oncogenic miRNA addiction for tumor cells [48].

MicroRNAs deregulation can be caused by several mechanisms including deletion, amplification, mutation or dysregulation of transcription factors that target specific miRNAs. In addition, miRNAs can be controlled by epigenetic mechanisms. In 2006, Saito *et al.* was the first to demonstrate that miRNAs expression could be controlled by the two major epigenetic mechanisms: DNA methylation and histone modifications [49,50]. When Saito *et al.* simultaneously treated a human bladder carcinoma cell line with 5-aza-2-deoxycytidine (a DNA methylation inhibitor) and 4-phenylbutyric acid (a histone deacetylase inhibitor), 17 out of 313 human miRNAs were found upregulated. In particular, miR-127 was upregulated after treatment with these drugs and expression of one of its targets, BCL6, was suppressed [49]. Accordingly, miR-127 expression was downregulated in primary human bladder and prostate tumors comparing to normal tissue [49]. Therefore, the authors concluded that in cancer tissue miR-127 is subject to epigenetic silencing [49,50]. This finding opened the field of epigenetics to miRNAs regulation. Interestingly, a bidirectional connection between epigenetic and miRNAs has been

established. On one hand, epigenetic mechanisms control miRNAs and, on the other hand, miRNAs can target essential epigenetic key players. One of the first reports suggesting methylation could be controlled by miRNAs was described in 2004 in *Arabidopsis* - Bao *et al.* suggests that miR-165 and miR-166 are required for the methylation at the *PHB* gene [50,51].

In cancer patients, metastasis is the principal cause of death. Metastatic process involves multiple steps: cell motility, invasion of adjacent stroma, intravasation, systemic dissemination (either though the blood or lymph), extravasation into the parenchyma of distant tissues and finally proliferation at a new site giving rise to secondary tumor. In this process miRNAs have a dual role as they can promote or inhibit metastasis [52]. The first sighting about miRNA function as metastasis activators was reported by Ma *et al* [53]. MiR-10b positively regulates cell migration and invasion *in vitro* and is capable of initiating tumor invasion and metastasis *in vivo*. MiR-10b acts by directly targeting HOXD10 which is a transcriptional repressor of RHOC, a key player in metastasis. Concordantly, miR-10b expression is elevated in about 50% of metastatic breast tumors compared with metastasis-free tumors or normal breast tissues [53]. On the same line, Huang *et al.* found that human miR-373 and miR-520c stimulated by suppression of CD44 cancer cell migration and invasion in vitro and in vivo [54].

In contrast, miRNAs can prevent tumor metastasis. Tavazoie *et al.* published the initial study of miRNAs as metastasis suppressors [55]. In breast cancer, patients with low expression levels of miR-335, miR-126 and miR-206 had a shorter median time to metastatic relapse. Restoration of their expression in breast cancer cell lines decreased number of metastasis in inoculated mice. These miRNAs have distinct mechanisms for metastasis suppression: restoration of miR-126 expression significantly suppressed overall tumor growth; while restoration of miR-335 or miR-206 levels altered cells morphology, possibly causing a decrease in cell motility [55].

It was not therefore surprising that Lujambio *et al.* reported in 2008 that DNA methylation-associated silencing of tumor suppressor miRNAs contributes to the development of human cancer metastasis and that the reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited their motility, reduced tumor growth, and inhibited metastasis formation in xenograft models, with an associated down-regulation of the miRNA oncogenic target genes, such as C-MYC, E2F3, CDK6, and TGIF2 [56].

microRNAs in cardiovascular diseases

In 2005, Zhao *et al.* reported that miR-1-1 and miR-1-2 were specifically expressed in cardiac and skeletal muscle precursor cells and that miR-1 regulates ventricular cardiomyocytes. MiR-1 overexpression specifically in the developing heart of mouse model leads to a decrease pool of proliferating ventricular cardiomyocytes [57]. In the same year, Kwon *et al.* found that miR-1 in *Drosophila* modulates cardiogenesis [58]. Subsequently, in 2006, Cheng *et al.* reported that miRNA-1 promotes myogenesis, while miR-133, clustered together with miR-1 on mouse chromosome 2, stimulates myoblast proliferation [59]. Theses three reports were the first to suggest a possible involvement of miRNAs in cardiac-related human diseases. Rooij *et al.* published the first report associating miRNAs to heart failure and cardiac hypertrophy [60], which occurs in response to stress and injury and is characterized by an increase in cardiomyocyte size with no change in myocyte number [61]. Using miRNA microarray analysis, more than 12 miRNAs were identified as deregulated during cardiac hypertrophy and heart failure [60]. Interestingly, overexpression of miR-195 led to heart failure on mouse model and to hypertrophic growth of cultured rat cardiomyocytes [60,61]. In the subsequent years, several studies demonstrated miRNA aberrant patterns in cardiac hypertrophy and analyzed their roles [61], including miR-21, which is also one of the most deregulated miRNAs in cancer [62,63], suggesting common miRNA pathways involved in both abnormal states.

microRNAs in autoimmune diseases

Psoriasis is the most prevalent chronic inflammatory skin disease in adults and the first inflammatory disease in which miRNAs were implicated [64]. In 2007, Sonkoly *et al.* described that miR-203, exclusively expressed in keratinocytes, is upregulated in psoriasis-affected skin compared with healthy human skin or another chronic inflammatory skin disease. Interestingly, miR-203 targets SOCS-3 [64]. It has previously been described that SOCS-3 suppression leads to sustained activation of STAT3 in response to IL-6 and that STAT3 constitutively activation in keratinocytes leads to spontaneous development of psoriasis in mice [64-66]. MiR-146 is the other miRNA Sonloky *et al.* found associated with psoriasis. It was found upregulated in psoriasis patients and it is absent from keratinocytes and dermal fibroblasts but it is expressed by immune cells [64]. The upregulation of a miRNA associated with inflammation is not surprising since several studies support a role of a deregulated immune system in psoriasis [67]. MiR-146

targets IRAK1 and TRAF6, both regulators of the TNF-alpha signaling pathway [68]. Treatments using anti-TNF agents, such as infliximab, etanercept or adalimumab are effective for psoriasis [69,70]. Therefore, future studies should address treatments with miR-203 and miR-146 antagomirs for psoriasis patients in order to improve their quality of life [64].

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by chronic inflammation of synovial tissue [71]. In 2008, three studies reported the first association studies between RA and miRNAs. Stanczyk *et al.* described increased expression of miR-155 and miR-146 in RA synovial fibroblasts and RA synovial tissue [72]; one month later, Nakasa *et al.* confirms the expression of miRNA-146 in RA synovial tissue and further describes its induction by stimulation with TNF and IL-1 [73]; and in the same year, Pauley *et al.* reported increased expression of miR-146 in rheumatoid arthritis peripheral blood mononuclear cells, which mimics the results found on synovial tissue/fibroblasts [74]. Taken together these results suggest that miR-146 can be used as a biomarker for RA. In addition, several other miRNAs have been implicated RA, such as, miR-155, miR-132, miR16 [75], miR-346 [76] or miR-223 [77].

In lupus erythematosus (SLE), an autoimmune disorder characterized by excessive production of a variety of autoantibodies against several self-antigens [78], few studies have been published about the differently expressed miRNAs. Nonetheless, miRNAs might be implicated in etiology of this disease and evidences suggest that miRNAs are potential diagnostic markers in SLE [79-82]. Dai *et al.* described for the first time 16 miRNAs differently expressed in SLE [79]. The authors were also the first to describe 66 miRNAs differently expressed in lupus nephritis (LN) patients [83]. Several studies in 2010 confirm the link between miRNAs and SLE/LN. Two miRNAs (miR-21 and miR-148a) are overexpressed in CD4+ T cells from patients with lupus. MiR-148a directly downregulated DNMT1 expression and miR-21 indirectly downregulates DNMT1 expression by targeting, RASGRP1 an important autoimmune gene. By causing DNMT1 downregulation, both miRNAs contribute to DNA hypomethylation in lupus CD4+ T cells [80]. Te *et al.* analyzed miRNAs in lupus nephritis patients from two racial groups (African American and European American) and concluded that 5 miRNAs (miR-371-5P, miR-423-5P, miR-638, miR-1224-3P and miR-663) were differentially expressed across different racial groups [81]. Recently, Zhao *et al.* showed that miR-125 expression was reduced and, accordingly, expression of its predicted target KLF13 was increased in SLE patients [82].

Although studies with larges number of samples are necessary, these studies implicate miRNAs in immune diseases pathogenesis and suggest that they can be used as potential diagnostic markers.

microRNAs in neurodegenerative diseases

A significant fraction of miRNAs is specifically expressed in the central nervous system and plays a role in neuronal development [84]. Consequently, it seems natural that miRNAs have been linked to neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease, which are caused by excessive neuronal death in the diseased brain [85]. In 2007, there were reported the first studies associating miRNAs to neurodegenerative diseases. Using Purkinje cells, Schaefer *et al.* suggested an involvement of miRNAs in neurodegenerative disorders by showing progressive neurodegeneration (cerebellar degeneration and development of ataxia) in the absence of the miRNA biogenesis key component, DICER, which consequently causes progressive loss of miRNAs [86,87]. Lukin *et al.* found that miR-9, miR-25b and miR-128 were upregulated, and that miR-124a was dowregulated, in Alzheimer brain samples when compared with aged-match controls [88,89]. In the same year, a miRNA important for maturation and function of dopaminergic neurons, miR-133b, was one of the first miRNAs reported to be lost in Parkinson's disease midbrain tissue [90].

MiRNA expression profiles for neurodegenerative diseases are challenging for two main reasons: 1) some miRNAs are expressed specifically by only some brain regions or brain cell types; 2) unlike cancer, it is much more challenging to obtain disease-affected and healthy tissue from the same patient, which turns more difficult to control these type of studies [91]. From 2007 till present, several studies have been linking miRNA modifications to regulation of proteins involved in these diseases [92]. Although studies seem to indicate that miRNAs can participate in neurodegenerative disease initiation or progression, more work is necessary to clarify if changes in miRNAs expression directly contribute to the pathogenesis of neurodegenerative diseases or if they are secondary events caused by deregulated pathways in these diseases [89]. It is predicted that in the next years, increase publications on miRNAs will let to novel insight in this field [91].

microRNAs as molecular biomarkers

In a clinical context, miRNAs can be extremely useful as disease diagnosis, prognosis and prediction of therapeutic response markers. In 2004, Takamizawa and coworkers were the first to pinpoint the prognostic value of miRNAs by showing that let-7 expression was reduced in lung cancers and that lung cancer patients presenting low let-7 expression levels have a significantly shorter survival after potentially curative resection [93]. In 2005, Calin *et al.* reported the first study showing the diagnostic/prognostic importance of miRNAs at a genomewide level [94]. The authors found that miRNA expression profiles could be used to distinguish normal B cells from malignant B cells in patients with CLL. In fact, a unique miRNA expression signature is associated with prognostic factors such as ZAP-70 expression (predictor of early disease progression) and mutational status of IgVh. In addition, the authors found 9 miRNAs differently expressed between patients with a short interval from diagnosis to initial therapy and patients with a significantly longer interval. Furthermore, this study also highlight that one mechanism of miRNA deregulation is mutation: a germline mutation in the precursor of miR-16-1-miR15a caused low levels of miRNA expression both *in vitro* and *in vivo* [94].

Currently, the clinical utility of miRNAs as diagnostic/prognostic biomarkers has been demonstrated in several types of cancer by numerous studies using tumor samples removed during surgery or biopsies [42]. However, for non-malignant diseases larger and more studies should be conducted.

microRNAs as biomarkers in plasma or serum

Current techniques for cancer diagnosis commonly involve a biopsy to the cancer tissue. Because this technique is invasive and unpleasant for patients, some studies have been focused on the search for biomarkers in human fluids such as plasma/serum, urine or saliva. Blood samples from patients are usually readily available and many biological molecules, as circulating nucleic acids, can be found in blood serum/plasma, including miRNAs [95,96]. These small non-coding RNAs in the blood are incorporated in microparticles and exosomes (50- to 90-nm membrane vesicles) that prevent its degradation, conferring an advantage to the use of miRNAs as markers in serum [95]. In addition, detection of miRNAs is serum is easy due to highly sensitive PCR detection methods, the lack of post-processing modifications of miRNAs, and simple methods of miRNAs extraction from serum [95]. The first report addressing the utility of miRNAs as diagnostic tools in biological fluids was published in 2008 by Chim *et al.* in a study

that detected placental miRNAs in the maternal plasma [97]. In the same year, Lawrie *et al.* [98], by comparing serum from diffuse large B-cell lymphoma patients and healthy controls, found that miR-155, miR-210 and miR-21 levels were significantly upregulated in patients. Interestingly, these miRNAs have been shown to be deregulated in tumors. Moreover, high expression of miR-21 in patients serum was correlated with improved relapse-free survival times [98]. To date, miRNAs deregulation in serum of cancer patients have been described in several tumors as leukemia, lymphoma, gastric, colorectal, lung, oral and squamous cell cancer, breast, ovarian, prostate, pancreatic and hepatocellular cancer [96].

Furthermore, a recent report analyzed miRNAs plasma levels in rheumatoid arthritis or osteoarthritis patients. Remarkably, plasma miR-132 was significantly higher in healthy controls than in rheumatoid arthritis or osteoarthritis patients and therefore it can be a potential diagnostic marker. In addition, plasma miRNA levels correlate with disease activity in rheumatoid arthritis. However, miRNAs included in this study failed to differentiate rheumatoid arthritis and osteoarthritis. Thus, others miRNAs specific for both diseases should be studied. Interestingly, this study also analyzes miRNAs present in another body fluid, namely in synovial fluid, and demonstrate that miRNAs from plasma and from synovial fluid have different origins [99,100].

In order to translate miRNAs expression serum evaluation into a clinical routine for diagnostic/prognosis practice, it is still necessary to standardize the methodologies used for these studies, namely, serum/plasma extraction procedures and storage conditions, housekeeping miRNAs for normalization in serum samples or the used of same statistical methods for data analysis [95]. In addition, large studies reporting miRNA levels in plasma and serum with detailed clinical data information, together with normal controls from both genders and different ages are still missing [96].

Therapeutic implications

MiRNAs are aberrantly expressed in several diseases; therefore, it is not surprising that these small ncRNAs represent potential therapeutic targets for the diseases they are functionally associated with. MiRNAs that are upregulated in diseases should be targeted using anti-miRNAs which are antisense oligonucleotides with specific modifications [101]. For instance, antagomirs, a class of anti-miRNAs that is cholesterol-conjugated to facilitate cellular intake and serum proteins binding, could be used to block oncomirs effect in cancer [101,102]. In 2004, Hutvagner

et al. successfully copy the phenotype of let-7 loss-of-function mutation by injecting a 2' O-methyl oligonucleotide complementary to let-7 miRNA into *C. elegans* larvae [103]. In 2005, Krutzfeldt *et al.* reports for the first time the use of antagomirs *in vivo* in mammalians [104]. In mouse model, Krutzfeldt and colleagues systemically deliver via intravenous injection antagomirs against miR-16, miR-122, miR-192 and miR-194 which specifically downregulated the corresponding miRNAs. Silencing of miRNAs using antoagomirs was long lasting and miR-16-antagomir effects were detected in multiple tissues, except in brain, possibly due to the blood-brain barrier [101,102,104,105]. Other approaches to efficiently inhibit miRNAs *in vivo* is the use of locked nucleic acid (LNA) oligos or 2'-O-methoxyethyl phosphorothioate (MOE) modification [102]. Elmen *et al.* evaluated for the first time the effect of an LNA-antimiR in non-human primates with surprising results. The authors intravenous injected a LNA-anti-miRNA-122 into African green moneys and were able efficiently silence the mature miR-122. The effect was long-lasting and safe, as toxicity associated to LNA as well as histopathological changes were not detected [106]. Two years later, Landford *et al.* described the utility of anti-microRNAs for the clinical pratice. MiR-122 is a liver-expressed miRNA essential for hepatitis C virus replication (HCV). Using a LNA-anti-miR-122, the authors were able to suppress HCV viremia in chronically HCV-infected chimpanzees. Moreover, this therapy generated a high barrier to resistance and no side effects were detected [107].

In addition to theses direct-inhibitory methodologies, an indirect technology can be used through downregulation of miRNA biogenesis pathway components. Tetracycline-inducible shRNAs could be used to downregulate Dicer or Drosha, key components of the miRNA-biogenesis pathway; however, this mechanism should be tightly controlled as downregulation of this pathway will have an effect on all miRNAs [101,102].

On the other hand, when miRNAs downregulation promotes disease, as it is the case of some miRNAs dowregulated in tumors which function as tumor suppressors, a therapeutic approach would be to restore the mature miRNA levels in the proper tissue/cells. In this situation, it should be used synthetic RNA duplexes, resembling siRNA molecules, that will mimics miRNAs duplex and will be recognized by RISC complex [102]. This complex will process it as the endogenous miRNA, by loading the stand with the sequence identical to the mature miRNA. This approach still needs to be evaluated *in vivo* since some challenges, such as stability and delivery strategies, need to be improved [101,102,108]. The use of short-hairpins

driven by Pol III promoters could be a strategy for re-expression of miRNAs in the cells [108]. Though, dosage used in this mechanism has to be narrowly controlled, since sustained shRNA high-level expression in livers of adult mice has shown to be fatal [109]. In 2009, Kota *et al.* demonstrated how useful systemic administration of miRNAs can be for anti-cancer therapy. MiR-26a is expressed at low levels in hepatocellular carcinoma but normally expressed in other tissues. The authors used an adeno-associated virus to mediate miR-26a delivery in a mouse model of liver cancer and were able to reduce cancer cell proliferation and induce tumor cells apoptosis, which consequently caused tumor regression. Since only cancer cells present miR-26a downregulation, the delivery was highly specific and didn't affect the normal tissue that was tolerant to miR-26a restoring [110].

Concluding remarks

MiRNAs were discovered in 1993 and rapidly became an exciting topic of research during the last decade with the number of publications growing exponentially. Like miRNAs, other ncRNAs have recently been linked to diseases [111, 112]. Therefore, we may predict that advances on understanding the roles of other classes of ncRNA-mediated regulation in diseases will be revealed at an extremely rapid rate in next years. In future years, it is expected that the acquired biological knowledge on miRNAs can be widely translated into the clinics. Despite major challenges that still need to be overcome (such as tissue specific delivery) miRNAs hold a great potential as therapeutic targets.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

Figure 1 – Historical perspective on the evolution of our knowledge about miRNAs. miR: microRNA; UTR: untranslated regions; CDS: coding sequences; LNA: locked nucleic acid