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Review

MicroRNAs: New Biomarkers for Diagnosis, Prognosis, Therapy Prediction and Therapeutic Tools for Breast Cancer

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Abstract

Dysregulation of microRNAs (miRNAs) is involved in the initiation and progression of several human cancers, including breast cancer (BC), as strong evidence has been found that miRNAs can act as oncogenes or tumor suppressor genes. This review presents the state of the art on the role of miRNAs in the diagnosis, prognosis, and therapy of BC. Based on the results obtained in the last decade, some miRNAs are emerging as biomarkers of BC for diagnosis (i.e., *miR-9*, *miR-10b*, and *miR-17-5p*), prognosis (i.e., *miR-148a* and *miR-335*), and prediction of therapeutic outcomes (i.e., *miR-30c*, *miR-187*, and *miR-339-5p*) and have important roles in the control of BC hallmark functions such as invasion, metastasis, proliferation, resting death, apoptosis, and genomic instability. Other miRNAs are of interest as new, easily accessible, affordable, non-invasive tools for the personalized management of patients with BC because they are circulating in body fluids (e.g., *miR-155* and *miR-210*). In particular, circulating multiple miRNA profiles are showing better diagnostic and prognostic performance as well as better sensitivity than individual miRNAs in BC. New miRNA-based drugs are also promising therapy for BC (e.g., *miR-9*, *miR-21*, *miR34a*, *miR145*, and *miR150*), and other miRNAs are showing a fundamental role in modulation of the response to other non-miRNA treatments, being able to increase their efficacy (e.g., *miR-21*, *miR34a*, *miR195*, *miR200c*, and *miR203* in combination with chemotherapy).

Key words: Breast cancer, microRNA/miRNA, circulating biomarker, theranostic, diagnosis, prognosis, prediction and therapy.

1. Introduction

In 1993, Lee et al. [1] described that a small non-coding RNA in *Caenorhabditis elegans* was able to regulate the expression and function of another protein-coding mRNA. The discovery of microRNAs (miRNAs or miRs) had a profound impact on the understanding of many gene regulation processes in the following years. Since they were first discovered, the physiological relevance of miRNAs in regulating plant and animal gene expression has been established.

The primary repository for miRNA sequences and annotations, miRBase (www.mirbase.org), debuted in 2006 with just 218 miRNA loci [2-4]. Since then, novel high-throughput sequencing techniques applied to miRNA analysis have allowed the discovery of more than 28000 mature miRNAs (miRBase release June 21, 2014). MiRNAs participate in the post-transcriptional regulation of gene expression in almost all key cellular processes [5], such as regulation of cell proliferation, differentiation, angiogenesis, migration, and apoptosis.

Significant evidence has accumulated in the last

few years, showing a fundamental role of miRNAs in the development of many diseases [6-9]. In particular, in cancer, aberrations in miRNA expression levels have been linked to the onset and progression of various types of cancer [10].

Breast cancer (BC) is the second most common cancer in the world, and by far, the most frequent cancer among women, contributing to an estimated 25% of all new cancers or cases diagnosed in 2012 [11]. Several biological features are routinely used for the diagnosis and prognosis of patients with BC and for determining the therapy, e.g., histological grade [12], lymph node status, hormone receptor status, and human epidermal growth factor receptor type 2 (HER2) status [13]. Some of these factors have been associated with the survival rate of patients and their clinical outcome after treatment [14]. However, some patients, with a similar combination of BC features, have been found to have different clinical outcomes. Thus, the role of these factors in determining diagnosis and prognosis and in predicting therapeutic outcomes in BC remains limited [15].

New affordable methods are therefore needed to help diagnosis and prognosis and to suggest the most appropriate treatment for patients with BC on an individual basis. As a solution, miRNAs have been proposed as promising biomarkers of BC because they can be readily detected in tumor biopsies (non-circulating miRNAs) [16, 17] and are also stably found in body fluids (circulating miRNAs), particularly in blood, plasma, serum, and saliva [18, 19]. These circulating miRNAs are highly reliable and protected from endogenous RNAse activity, being bound to lipoproteins such as HDL, associated with Argonaute 2 (Ago2) protein [20], or packaged into microparticles (such as exosome-like particles, microvescicles, and apoptotic bodies.) [19, 20].

Recently, miRNA profiling has been assessed to improve BC classification and to differentiate patients with BC as responding or not responding to therapies, with promising results [21]. It is now clear that these tools have the potential to provide new diagnostic, prognostic, and predictive biomarkers for BC, with a great impact on the clinical management of patients with BC [15].

In this review, we focused on the recent findings related to the role of miRNAs in BC and on how miRNAs have the potential to answer actual clinical needs, such as identification of biomarkers for early and differential diagnosis, prognosis, and prediction of response to specific therapies. New therapeutic strategies represented by miRNA-based theranostic approaches in BC are also introduced and could become a starting point for the future development of novel therapeutic tools.

2. miRNA biogenesis and mechanisms of action

2a. miRNA biogenesis

miRNAs are small, evolutionarily conserved, non-coding RNAs that are approximately 18–25 nucleotides in length and constitute the dominating class of small RNAs in most somatic tissues. Other small RNAs in animals include silencing RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), which are typical of germinal cells [22], and non-coding mitochondrial RNAs (ncmRNAs) [23]. Although many aspects of the miRNA biogenesis pathway and repressive mechanisms are still obscure, the key processes have been fully characterized.

miRNAs are transcribed from individual genes containing their own promoter, or intragenically from spliced portions of protein-coding genes [24]. Like protein-coding genes, miRNAs with their own promoters are almost exclusively transcribed by RNA polymerase II in a primary transcript called pri-miRNA [24] (Figure 1). This long transcript contains a 7-methylguanosine cap at the 5′ end, a 3′ poly-(A) tail, and sometimes also introns. To be processed, pri-miRNAs are recognized by Drosha ribonuclease and its partner, the double-stranded RNA binding protein DGCR8, through interaction with a stem–loop structure within the miRNA in which the sequences are not perfectly complementary [25, 26]. Processing of pri-miRNAs gives rise to precursor miRNAs (pre-miRNAs) of approximately 70 nucleotides [24] (Figure 1). Some intronic miRNAs, called mirtrons, could bypass Drosha processing and use the splicing machinery to generate pre-miRNAs [24]. The generated pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin 5 (XPO5) [27-29], where they are cleaved by the RNase III enzyme Dicer 1 in union with transactivation-responsive RNA-binding protein 2 (TARBP2) and AGO2 (DICER complex). The processing generates a double-stranded miRNA–miRNA* duplex [30]. The 2 strands are then separated: the mature miRNA (the guide strand) is incorporated into the RNA-induced silencing complex (RISC), whereas the passage miRNA* strand can be loaded in the RISC as well or degraded [31-33]. The mature miRNA guides the AGO protein of the RISC to the complementary mRNA sequence on the target to repress its expression [24] (Figure 1).

2b. miRNA mechanisms of action

The major determinant for miRNA binding to its target mRNA is a 6–8-nucleotide sequence at the 5′ end of the miRNA, the "seed" sequence [24]. Any sequence complementarity between the loaded

miRNA and the seed region triggers a detectable decrease in target mRNA expression levels. Seed matches can occur in any region of the mRNA but are more likely to be present in the 3′ untranslated region (3′ UTR) of a mRNA [34, 35]. Several lines of evidence indicate that miRNAs can also bind to other regions in the target mRNA [36]. Depending on the degree of homology to the 3′ UTR target sequence, miRNAs can induce the translational repression or degradation of mRNAs. Given that each miRNA is capable of regulating the expression of many genes, each miRNA can simultaneously regulate multiple cellular signaling pathways.

Apart from the "traditional" mechanism of action of miRNAs described above, other "non-canonical" mechanisms have been proposed recently. Some evidence indicates that miRNAs could increase the translation of a target mRNA by recruiting protein complexes at the AU-rich region of the target mRNA or they could indirectly increase target mRNA levels by interacting and modulating repressor proteins that block the translation of the target mRNA [37]. Other evidence suggests that miRNAs could enhance ribosome biogenesis, thereby modulating protein synthesis, or skip cell cycle arrest, thereby activating target gene repression [34, 38].

3. Methods for miRNA target prediction and miRNA–target interaction validation

3a. Methods for miRNA target prediction

Uncovering of miRNA-regulated networks needs large-scale and unbiased methods for miRNA target identification. For instance, the differential expression of a single miRNA would be followed by downstream gene or proteome-wide analysis. A single miRNA could regulate a set of genes responsible for a particular malignant phenotype. The silencing of that single miRNA can alter the entire set of genes.

To overcome this complexity and to predict the target genes, several algorithms have been developed. The main difficulty in miRNA target prediction is to detect the specific sequences within genes where one miRNA is fully or partially complementary [39], considering the small size of miRNAs and their low specificity.

A collection of tools is available, each with a distinct approach to miRNA target prediction and different features [40]. The suitable tool can be decided depending on the requirements [12].

The major features of computational target prediction are as follows: sequence composition (e.g., seed match), conservation, and thermodynamic stability (e.g., free energy).

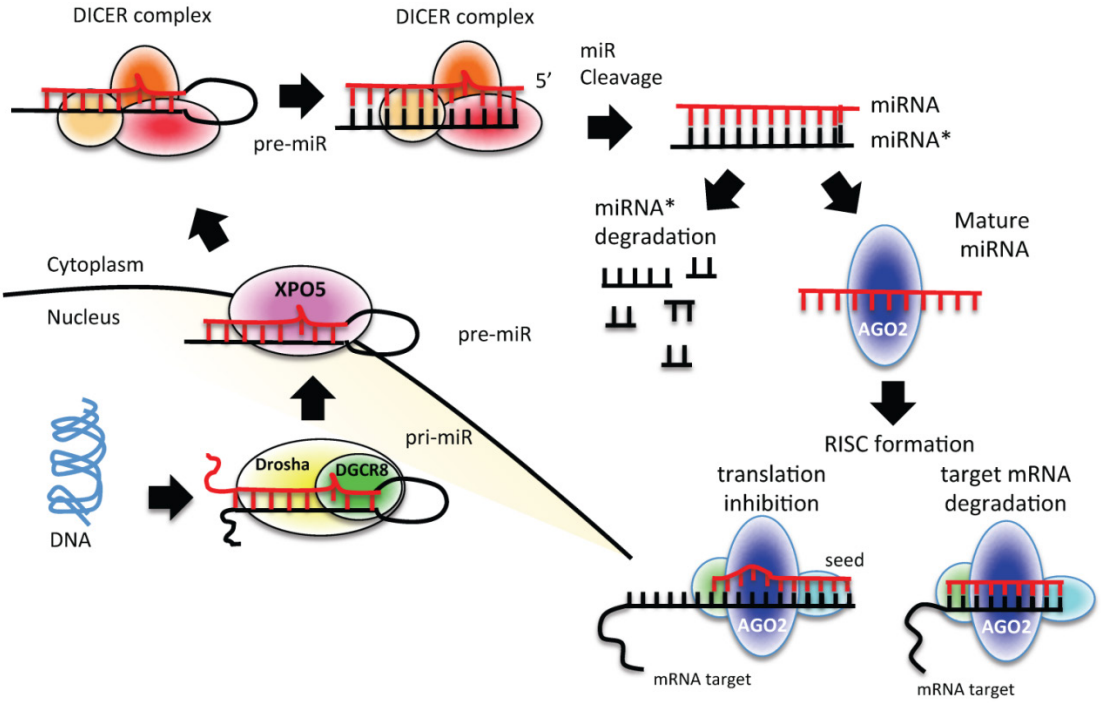

Figure 1: miRNA biogenesis process. A schematic representation of canonical miRNA biogenesis pathway. Each miRNA is transcribed by RNA polymerase II (pri-miRNA) from genomic DNA within the nucleus; pri-miRNA is recognized by Drosha-DGCR8 and processed to pre-miRNA. Pre-miRNA is exported to the cytoplasm by exportin 5 (XPO5), where it is processed and cleaved by DICER complex to a double strand miRNA (miRNA*-miRNA). The duplex is cleaved, and only the mature miRNA is loaded into the RISC complex. The degree of homology of the miRNA "seed" to the 3′ UTR target sequence of the mRNA determines the mRNA translational repression or degradation.

i) Seed match is the start of many computational methods for miRNA target prediction. A seed match usually consists of Watson–Crick (WC) complementarity between the miRNA and miRNA target nucleotides. WC complementarity occurs when adenosine (A) pairs with uracil (U) and guanine (G) pairs with cytosine (C). The seed is a sequence from the 1st to the 8th nucleotide at the 5′ end of the miRNA. However, algorithms based only on WC complementarity show low accuracy and a high number of false-positive results [26].

Other sequence compositions can be used as features for miRNA target prediction tools. Bartel et al. [31] showed that the AU residues in target sites improve the accessibility of miRNAs to form duplexes. Recent studies have suggested that coding regions of mRNAs can also include target sites for miRNAs [41]. In addition, it has been demonstrated that a transcript can contain multiple target sites for a single miRNA; however, when the target sites show overlapping sequences, miRNA–mRNA pairing can be compromised [42].

ii) Conservation analysis was introduced in order to reduce false-positive results. Conservation refers to the maintenance of sequence homology across species [40]. In general, there is higher conservation in the miRNA seed region than in the non-seed region [43]. However, the limit of this approach was demonstrated by Bentwich et al., who showed that several non-conserved miRNAs were missing [43].

iii) Free energy (or Gibbs free energy) can be used as a feature for miRNA target prediction [44, 45]. The thermodynamic stability of the miRNA–mRNA duplex shows the strength of the binding between a miRNA and its target by predicting how the miRNA and its candidate target will hybridize. The free energy is related to duplex formation between the miRNA and its target site. In particular, pairing can be determined by removing existing secondary structures [46]. The free energy is established by the difference between the energy expended in opening the target site structure and that gained by forming the duplex [46, 47].

Many computational algorithms have been developed and implemented as software tools for miRNA target prediction using some of the described features. These packages are very useful to select putative miRNA targets for further biological validation. The most common classifiers are based on machine learning algorithms, e.g., support vector machine (SVM), neural networks, hidden Markov model (HMM), and Naive Bayes (NB). These machine learning methods are trained on a so-called "training" dataset that contains a set of known miRNA sequences (positive training dataset) and a set of sequences that do not contain miRNAs, such as mRNAs, tRNAs, and rRNAs (negative training dataset), which represent the limit of this approach [47]. Several studies have tried to overcome this problem with the use of only true/positive models [48-50]. However, the results are worse than those obtained with approaches that utilize both positive and negative training sets [49]. Many tools of machine learning-based approaches for miRNA target prediction are currently available, e.g., HHMMiR [51], PicTar [52], MiRFinder [53], RNAmicro [50, 54], ProMiR [55], MiRRim [56], BayesMiRNAFind [57], and SSCprofiler [58].

Other computational algorithms use approaches different from machine learning. The TargetScan algorithm was the first miRNA target prediction tool for human genome [40]. It searches for perfect complementarity in the seed region, and all seed sequences outside complementarity are filtered out. Predictions are ranked by a combinatorial score on the basis of sequence composition (seed sequence), conservation, and thermodynamic stability (free energy).

Diana-microT uses a larger frame for scanning complementarity. It focuses on orthologous human and mouse 3′ UTRs from the mRNA Reference Sequences (RefSeq) database and 94 miRNAs conserved in human and mouse. It applies a modified dynamic programming algorithm to calculate the minimum free energy for each segment with a miRNA [59].

The miRanda algorithm gives scores for seed complementary regions. The results are evaluated for free energy. Each target that has a predicted free energy below a threshold is then passed to the last step, i.e., conservation [60].

These algorithms are summarized in Table 1, together with their main characteristic approaches and features.

3b. miRNA–target interaction validation

Many experimental technologies for validating miRNA–mRNA interactions have been developed [61, 62]. In general, the effects of differential miRNA expression on the target gene obtained through transfection of miRNA mimic or miRNA inhibitor oligonucleotides or constructs [63] are established at the protein level by western blotting and at the mRNA level by quantitative real-time PCR (qRT-PCR), with a specific probe for the target gene [61, 62]. The most important disadvantage of these techniques is that they are not able to distinguish between direct and secondary miRNA–target interactions.

3b.1 Luciferase assay

Reporter assays are commonly used to study gene expression coupled with other cellular events,

such as receptor activity or intracellular signal transduction of protein–protein interactions. To analyze direct miRNA–mRNA interactions, the firefly luciferase-based assay is widely used because the reporter activity is available immediately upon translation, the assay is very rapid and sensitive, and no background luminescence is found in the host cells (Figure 2). To be used as a reporter assay for validation of the interaction of a miRNA with the 3′ UTR of a gene of interest (GOI), the luciferase-based assay needs cloning of the 3′ UTR of the GOI, where the miRNA-recognized sequence is supposed to be present, downstream of the luciferase gene in the reporter vector (Figure 2). The cells are then transfected with this construct in the presence or absence of the miRNA mimic oligonucleotide. If the miRNA is able to recognize the seed in the 3′ UTR of the GOI, the level of luciferase expression is decreased, thus causing a diminished bioluminescence emission (Figure 2B); on the other hand, if the miRNA does not interact with the 3′ UTR, the emission of light is unaffected (Figure 2A). The disadvantages of this type of reporter assays are that they are laborious, expensive, sensitive only for the 3′ UTR chosen for cloning, and difficult to use for transfection [62, 63].

Table 1. The main algorithms for computational miRNA-target prediction

Algorithm	Features	Approach	References
HHMMiR	Seed match, and conservation	HMM	[51]
PicTar	Seed match	HMM	[52]
MiRFinder	Seed match, and conservation	SVM	[53]
RNAmicro	Sequence composition, conservation, and thermodynamic stability	SVM	[54]
ProMir	Sequence composition, conservation and thermodynamic stability.	HMM	[55]
MiRRim	Sequence composition, conservation, and free energy.	HMM	[56]
BayesMiRNAFind	Sequence composition and free energy.	Naïve Bayes Classifier	[57]
SSCprofiler	Sequence composition, conservation and free energy.	HMM	[58]
Diana-microT	Seed match, conservation, and free energy	Dynamic programming algorithm	[59]
TargetScan	Seed match, conservation, and free energy	Combinatorial score	[40]
MiRanda	Seed match, conservation, and free energy	Score	[60]

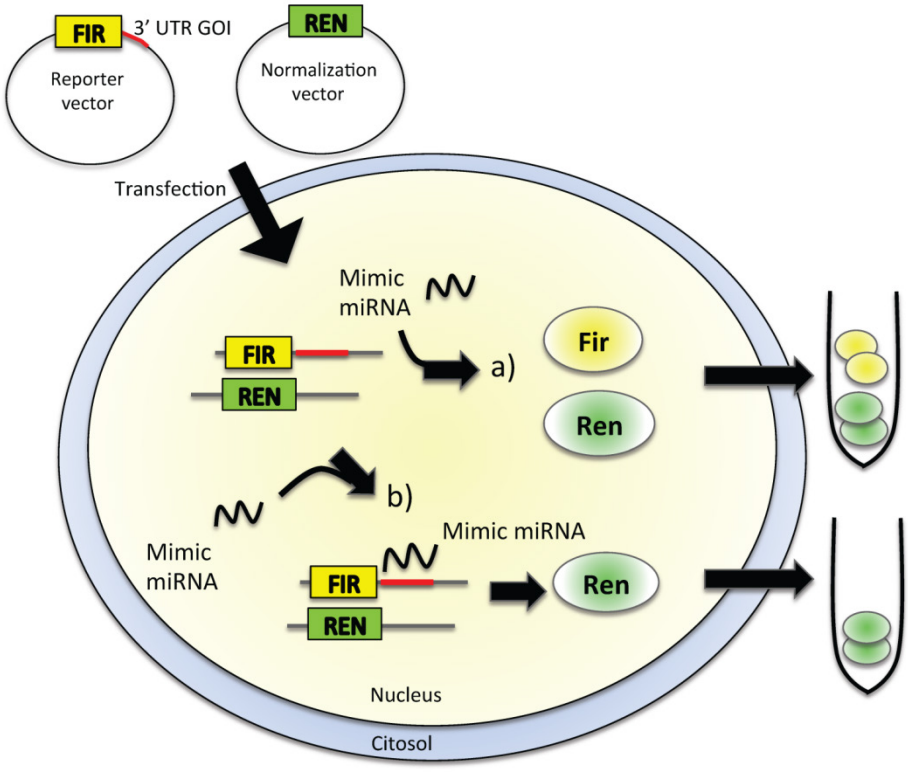

Figure 2: *In vitro* validation of miRNA-target direct interaction. Cultured cell lines are transfected with a reporter vector containing firefly (FIR) luciferase gene and the 3′ UTR of the gene of interest (GOI). The level of expression of FIR luciferase is measured in a luminometric assay. Cells are then exposed to the mimic miRNA, which is supposed to enter within the cell and to interact with the 3′ UTR of the GOI. If no interaction between miRNA and the 3′ UTR of GOI happens (a), we could observe no alteration in the level of expression of luciferase, thus no alteration in the emitted chemoluminescence, as FIR gene produced an active, luminescent protein. The complete interaction between the miRNA and the 3′ UTR of the GOI (b) leads to reduced FIR luciferase expression, with a decrease of luminescence levels. Other luminescent genes, such as Renilla (REN) luciferase, are usually used as reference genes for luminescence normalization.

3b.2 RISC immunoprecipitation

Another biochemical method to identify and isolate direct miRNA–target complexes is based on the immunoprecipitation of RISC components (such as AGO and TNRC6). This method is able to capture low-abundant and transient miRNA–mRNA pairs. Target mRNAs undergoing direct miRNA regulation are co-immunoprecipitated along with the RISC and are identified by qRT-PCR, microarray, or deep sequencing [64]. The successful pull-down of the entire complex relies on the strong interaction between the miRNA–target complex and RISC and on the ability of the used antibody to precipitate AGO2, the core RISC protein usually used for complex immunoprecipitation. Some companies have developed a dominant negative mutant of an RISC protein subunit to trap the miRNA–target complex into the RISC, thus limiting further processing [65]. This strategy allows the recovery of transient and low-abundance mRNA targets that would otherwise be lost. A FLAG epitope is then used for the capture of the entire complex [65]. qRT-PCR or next-generation sequencing techniques are used to confirm the interaction between the miRNA and the target mRNA.

4. miRNAs and BC

Advanced technologies, such as microarray expression data, have shown that aberrant miRNA expression is the rule rather than the exception in BC [66, 67]. The tight integration of miRNAs in physiological circuits could become a problem, because the dysregulation of a small number of miRNAs could profoundly affect the expression profile of the cells, driving them toward transformation [68]. BC miRNAs, which have an important role in the pathophysiology of the disease, facilitating invasion, metastasis, epithelial to mesenchymal transition (EMT), and maintenance of BC stem cells, have become an interesting topic in BC management.

4a. Mechanisms altering miRNA expression levels

Because of amplification, each miRNA can increase the control over its target gene. If the target gene is an oncogene, the cancer does not develop (*oncosuppressor-miRs)*; if the target gene is a tumor suppressor, the cancer develops (*oncomiRs)*. Due to deletion, each miRNA can reduce the control over its target gene. If the target gene is an oncogene, the cancer develops (*oncomiRs)*; if the target gene is a tumor suppressor, the cancer does not develop (*oncosuppressor-miRs)*.

Several mechanisms can influence miRNA expression levels (Figure 3). Tumors often present altered levels of mature miRNAs [101] as a consequence of the following:

1. Epigenetic mechanisms (Figure 3, section 1). A large proportion of miRNA loci on the genome are associated with CpG islands, giving strong bases for their regulation by methylation (Figure 3, section 1) [69]. A recent critical review on aberrant DNA methylation of miRNAs in BC showed that although aberrant DNA methylation is a well-described mechanism for gene silencing, an actual demonstration of the link between miRNA expression and gene methylation was still missing in several of the analyzed studies [70]. However, Castilla et al. have clearly demonstrated in 70 BC cases that a relationship exists between *miR-200* family expression, gene methylation, and metastatic potential of the tumors [71]. A mapping-based study has identified miRNA promoters silenced in BC [72], and different patterns of methylation have been observed in the *miR-200b* cluster promoter in different BC sub-types [72]. Aure et al., focusing their attention on *let7e-3p* miRNA, found that the genomic region that encodes for this miRNA belongs to a hypomethylated, and thus silenced, chromosome [73]. The researchers have associated *let-7e-3p* downregulation with poorer BC prognosis [73]. Another epigenetic phenomenon altered in BC is histone acetylation. Studies with deacetylase inhibitors have revealed that the reduction of acetylated histones could diminish the expression of anti-oncogenic miRNAs [74, 75].

2. A genetic alteration (Figure 3, sections 1 and 2), i.e., frameshift mutations resulting from microsatellite instability. Such genetic alternations can affect the expression of several mRNAs, e.g., the mRNA of TARBP2 (Figure 3, section 5), the Dicer stabilizing protein. This has been found, for example, in colorectal and gastric cancer [76] and in BC [77]. Moreover, more than half of the known miRNAs are located in cancer-associated region, such as fragile sites, minimal regions of loss of heterozygosity, minimal regions of amplification (minimal amplicons), or common breakpoint regions [78]. In the literature, some miRNA families emerge to be overall more involved in tumor development [79], such as the *let-7* miRNA family. In BC, several *let-7* family members, together with *miR-125b*, *miR100,* and *miR34a*, have been found to be located at fragile sites of human chromosomes (11q23–q24D), potentially contributing to aberrant *miRNA* expression [78].

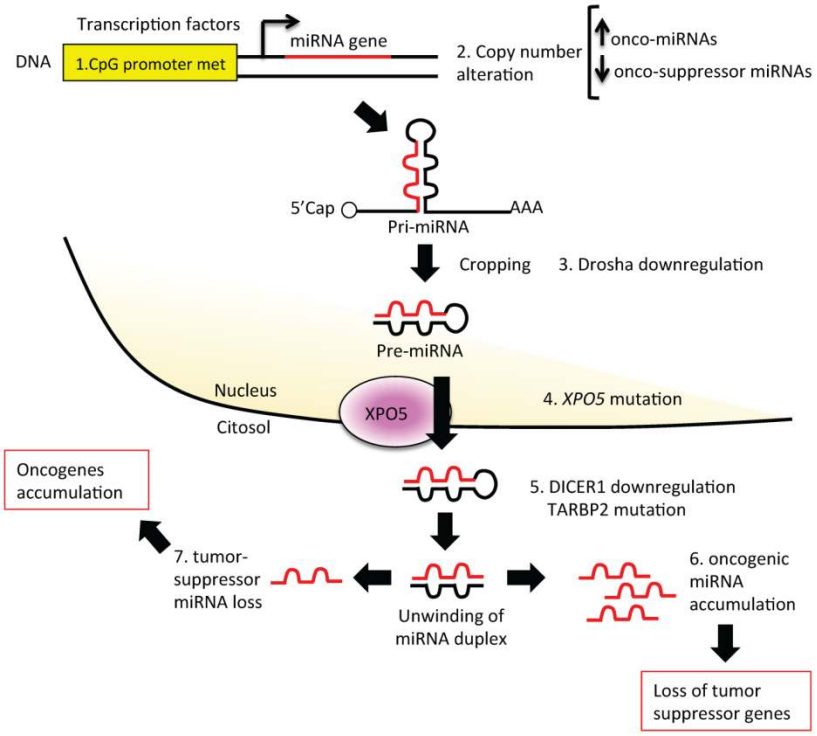

Figure 3: Altered steps in miRNA biogenesis lead to cancer. A schematic representation of altered steps of the miRNA biogenesis pathway, commonly deregulated in cancer: 1. miRNA genes contain upstream regulator elements (enhancers/repressors) and promoter regions, indicating that miRNAs are subjected to CpG methylation (CpG promoter met); 2. The alteration in the copy number of miRNA (due to genomic amplification or deletion, activating or repressing mutation, loss of epigenetic silencing and transcriptional activation) could increase the oncogenic miRNAs or decrease the tumor suppressor miRNAs; 3. Alteration in the miRNA processing machinery, i.e. downregulation of Drosha, could decrease the cropping of pri-miR to pre-miR; 4. XPO5 mutation could prevent pre-miR export to the cytoplasm; 5. Mutation of TARBP2 or downregulation of DICER1 decrease mature miRNA levels, causing finally a loss on tumor suppressor miRNAs; 6 and 7. Accumulation of oncogenic miRNAs or loss of tumor suppressor miRNAs could finally lead to cancer development.

3. Defects in the miRNA biogenesis pathway (Figure 3, sections 3–5): each step of miRNA biogenesis could be affected, thus altering miRNA expression levels and making the cell suitable for oncogenic changes. Reduced Dicer and Drosha expression (Figure 3, sections 3 and 5) have been associated with high-grade BC and shorter metastasis-free survival or with higher-grade BC and shorter disease-free survival [80-83]. Reduced Dicer expression (Figure 3, section 5) has been also found in many other human tumors [84], e.g., in prostate [85], gastric [86], or squamous cell carcinoma [87]. In BC, reduced Dicer expression has been associated with the triple-negative phenotype [83, 88]. Moreover, in BC, nucleolin (NCL), a component of the Drosha/DGCR8 microprocessor complex, has been demonstrated to promote the maturation of a set of metastasis-promoting miRNAs (*miR-221/222* cluster, *miR-21*, *miR-103,* and *miR-15a/16*) [89, 90]. Furthermore, XPO5, a key protein for pre-miRNA export to the cytosol, has been suggested as a possible prognostic biomarker for BC [91] (Figure 3, section 4).

4. Transcriptional repression by other upstream proteins (Figure 4). A plethora of transcription factors can influence the expression levels of a single miRNA. Several lines of evidence suggest that miRNAs and transcription factors work cooperatively. miRNAs are involved in the functional feedback loop, in which transcription factors influence miRNA expression levels and vice versa [92-94]. Thus, tumorigenic miRNA expression alterations could be due to the activity of tumor-related transcription factors, such as SMAD [90, 95], p53 protein family (p53, p63, and p73) [96], ataxia telangiectasia mutated (ATM) [97], and Myc [98]. In BC, the BC 1, early onset (BRCA1) transcription factor [99] and the epidermal growth factor receptor (EGFR/HER1), a hypoxic transcription factor involved in the regulation of the RISC [100], are able to inhibit miRNA maturation, thus enhancing cell survival and invasiveness.

4b. miRNAs and BC progression models

Modeling cancer disease is not easy, because cancer encompasses several histopathologies, involving genetic and genomic variations, and distinct clinical outcomes. A major challenge in advancing the knowledge of cancer is the availability of a single experimental model system that recapitulates the complex biology of the disease. Because of this complexity, no single model would be expected to mimic all features of the disease. The existing experimental models include two-dimensional (2D) and three-dimensional (3D) cell line cultures, xenografted mice, and engineered mice.

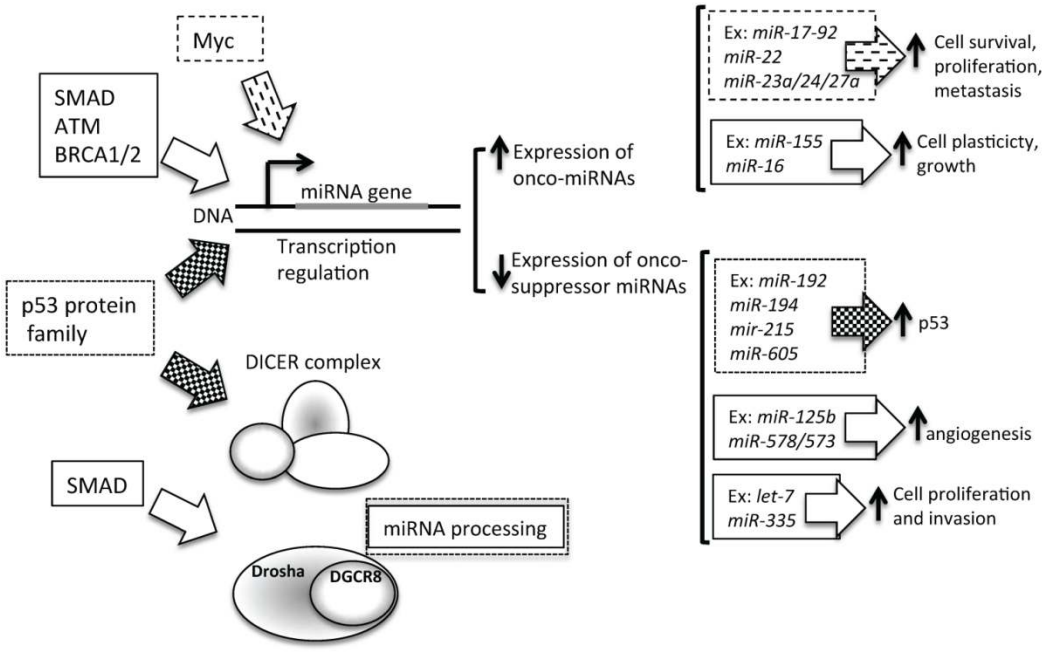

Figure 4: Contribution of transcription to miRNA level alteration in cancer. Several transcription factors are able to control the level of expression of miRNAs. In particular, as described in the text, SMAD, Myc, ATM, BRCA1/2 and p53 influence miRNA transcription. P53 can regulate onco-suppressor miRNAs, which are involved in the control of p53 turnover. SMAD, ATM, BRCA1/2 and Myc could influence the transcription levels of miRNAs involved in cell plasticity, cell proliferation and survival, and cell invasion control. Moreover, SMAD is also involved in miRNA processing, by Drosha expression levels control. Ex: example of miRNA regulated by transcription factors.

1. 2D cell culture. 2D cell culture studies in the oncogenic field have played a pivotal role in furthering our understanding of the disease mechanisms and drug discovery. The majority of scientific studies on miRNAs use 2D cell cultures for modulation of single miRNA expression and validation of the interaction between a single miRNA and its predicted targets via protein or gene expression analyses. This culture condition is easy to be manipulated, less expensive than the other approaches, and particularly suitable when a small number of miRNAs have to be studied.

Recently, particular attention has been given to emerging inadequacies associated with 2D culture systems, such as their inability to fully emulate *in vivo* tumor growth conditions and to provide physiological relevance. In fact, in the body, nearly all cells reside in an extracellular matrix (ECM) consisting of a complex 3D architecture, and interact with neighboring cells through biochemical and mechanical cues. These features cannot be obtained in 2D culture conditions. Cell–cell and cell–ECM interactions establish a 3D communication network that maintains the specificity and homeostasis of the tissue and influences tumor growth and its interaction with the whole organ. This approach has been extensively used in many works on the assessment of miRNAs in BC [101-103].

2. 3D cell culture. To overcome some shortcomings of 2D cultures, 3D cell cultures have been developed, with the use of specific matrix (such as natural ECM-based hydrogels, 3D spheroids, and trans-well inserts) that are able to support the growth of tumor cells for the establishment of physiological cell–cell and cell–ECM interactions of the native tissues. These matrix supports can mimic the environmental conditions in which the tumor cells grow with greater physiological relevance than conventional 2D cultures. The development of new biological supports is further fueled by the optimism that 3D models may significantly accelerate translational research in cancer biology. For example, use of 3D tumor cell culture is emerging as an important tool to characterize the morphogenesis of mammary epithelial cells and to elucidate the tumor-modulating actions of ECM. Focusing on miRNAs, the comparative analysis of 2D and 3D cell cultures has revealed a profound difference in miRNA profiles between the 2 culture conditions, particularly for BC cells and lung adenocarcinoma [104, 105]. In particular, the miRNA profiles in 2D and 3D cultures of 2 BC cell lines were compared. The findings revealed that the 3D culture exhibited a greater discrimination between the miRNA profiles than the 2D culture [105]. For example, the lower expression of *miR-429* was highlighted in the 3D culture-specific miRNA profile better than that in the 2D culture-specific profile, correlating with the 3D invasive capacity of the MDA-MB-231 BC cell line.

3. Xenografted mouse models. This approach takes advantage of the injection of cancer cells from human immortalized cancer cell lines or tumor cells from patients into mouse tissue to study the development of the tumor in its native environment. This

method can be used to obtain a miRNA profile during ongoing tumor development. Otherwise, tumor-xenografted mice are used for the therapeutic study of miRNA modulation. In fact, several reports have examined the effect of miRNA modulation by treating xenografted animals with oligonucleotides that increase (miRNA mimic) or decrease (antagomiR) the expression levels of a specific, single miRNA or by using an expression vector for miRNA level modulation [106]. Thus, the effects of miRNA modulation are analyzed by measuring the growth of the tumor, its invasive capacity, presence of metastatic masses, and vascularization of the tumor [107].

Several models to study miRNA involvement in BC progression are based on BC cell lines or patient specimens implanted into mouse mammary fat pads. For example, use of the triple-negative MDA-MB-231 BC cell line to generate a xenografted BC model allows identification of *miR-124* as the key regulator of the myc/p27/phospho-Rb pathway, which is usually altered in BC and ovarian cancer [108]. Another BC model, obtained by the orthotopic implantation of xenografted human BC specimens into NOD/SCID mouse mammary fat pads (called patient-derived human-in-mouse breast tumor xenograft model or PDX model), was used to study the spontaneous generation of BC-derived lung cancer metastasis. With a combined approach of gene expression arrays and global miRNA analysis, *miR-138* was demonstrated to be a key regulator of tumor invasion in lung, targeting the EMT process of BC cells [109]. Another xenografted mouse model was used to demonstrate the anti-metastatic potential of the peptide nucleic acid (PNA)-modified antagomiR-21 oligonucleotide on BC cells. Yan et al. [110] demonstrated that use of the antagomiR-21 oligonucleotide is able to block proliferation, cell migration, and *in vivo* tumor growth of 2 BC cell lines (MCF7 and MDA-MB-231) implanted in BALB/c-nude mice, proposing the use of this oligonucleotide for potential therapeutic applications in BC treatment.

4. Engineered mouse models have been widely used. These models could be of 2 types. The first type, the genetically engineered mouse model, obtained by oncogene amplification or tumor suppressor gene deletion to characterize a specific cancer, is used to generate an expression profile of miRNAs to clarify which miRNAs are involved in the development of that specific tumor. In BC, this model has been used to identify a miRNA profile associated with 8 different mammary-engineered mouse models [111]. In the second type, knocking-in or -out of specific miRNAs in the mouse germline allows to study the influence of miRNAs on tumor progression [112]. This approach also involves using genetic constructs to induce miRNA overexpression or downregulation in a particular tissue, at a particular development stage, or under pharmacological control. For example, several strains of mice lacking or overexpressing cancer-associated miRNAs have been developed and characterized. These include germline transgenic or knockout mice for the following: *miR-155,* which, if overexpressed in B cell lineage, induces B cell malignancy [113]; *miR-21*, which leads to lung tumorigenesis if ubiquitously deleted [114]; *miR-17-92* and its paralogs, whose overexpression in lymphocytes induces lymphoproliferative disease and autoimmunity [115]; *miR-15* and *miR-16*, whose deletion induces lymphoproliferative disorders [116]; *miR-146*, which causes myeloid sarcomas and lymphomas when deleted [117]; and *miR-29*, whose deletion causes B-cell lymphoma [118].

To our knowledge, no germline transgenic miRNA-engineered mouse models have been proposed yet to study BC onset and development.

5. miRNAs and cancer stem cells (CSCs)

5a. miRNAs involved in CSCs

The most recent definition of CSCs or tumor-initiating cells (TICs) identifies these cells as "a small subset of the cancerous population responsible for tumor initiation and growth, which also possesses the characteristic properties of quiescence, indefinite self-renewal, intrinsic resistance to chemotherapy and radiotherapy, and the capability to give rise to differentiated progeny" [119]. Chemotherapeutic agents kill differentiated tumor cells, but CSCs are generally unharmed. The existence of CSCs propels resistance to chemotherapy, disease progression, and disease relapse. The origin of CSCs is still an ambiguous issue. The main hypotheses regarding the origin of CSCs include the following: i) malignant transformation of normal stem cells, ii) de-differentiation of mature cancer cells through EMT, and iii) induction of pluripotent cancer cells. Leukemia stem cells (LSCs) in acute myeloid leukemia (AML) are the first and best characterized CSCs, often providing a working template for other types of cancers [120]. Similar to normal hematopoietic stem cells (HSCs), LSCs have extensive self-renewal property and are responsible for the maintenance of the bulk of leukemia blasts [120]. The somatic stem cell hypothesis describes the process where a dormant stem cell present in the adult organism could be transformed to a CSC because of a mutation or inappropriate regulation of stem cell pathways. The transformation of an adult stem cell (ASC) into a CSC could be due to epigenetic reprogramming processes (alteration in the DNA methylation of CpG islands, leading to the expression or si-

lencing of specific genomic regions; alteration in chromatin remodeling, which controls the accessibility of chromatin to transcription factors; and alteration in specific miRNA expression levels) [121].

The miRNA profile of CSCs is remarkably different from that of non-stem cancer cells, and many miRNAs have been shown to regulate the self-renewal and differentiation properties of CSCs [122, 123], such as the *let-7* miRNA family [124]. Being a tumor suppressor, the tumorigenic potential of the *let-7* family is due to its downregulation in many tumors, such as lung cancer or BC [125]. Cancer initiation, progression, and aggressiveness are driven by CSCs [126-128]. The *let-7* miRNA family appears to play a substantial role in the CSC phenotype. In fact, it seems that each tumor, being either hematologic or solid, includes a minor population of CSCs, capable of tumor initiation [129]. These TICs have downregulated *let-7* expression and, having tumor stem cell properties, can also undergo asymmetric division, thereby sustaining differentiated tumor proliferation [130]. In BC, *let-7* is found to be downregulated. In normal tissue, it plays the role of a regulator of self-renewal, acting as a pro-differentiation miRNA, whereas in BC it is repressed by the Wnt/β-catenin pathway [124]. Thus, its loss in BC leads to an increase in the CSC population.

In addition to *let-7*, *miR-34* has been described as a regulator of the Notch signaling pathway, necessary for stem cell maintenance, in colon CSCs [131]. Asymmetric cell division, a characteristic of CSCs required for self-renewal, is directed toward symmetry by the presence of *miR-146a*, which targets Numb to stabilize β-catenin expression and leads to symmetrical division [132]. In BC, expression of *miR-34* leads to cell cycle arrest [133], whereas its downregulation increases the invasive capacity and metastatic potential of BC cell lines *in vitro* and *in vivo* [134]. All the discussed miRNA as summarized in Table 2.

5b. miRNAs and EMT

Emerging evidence demonstrates that miRNAs play an essential role in controlling stem cell properties, such as self-renewal and differentiation, by regulating the expression of certain key stem cell regulatory genes [135-137] and by regulating EMT [138, 139]. EMT refers to the process in which tumor epithelial cells acquire mesenchymal features, with high invasiveness and metastatic abilities. In fact, EMT is associated with the loss of intracellular junctions and epithelial polarity and increase in cell motility, which are fundamental characteristics for tumorigenesis, invasion, and metastasis that allow cancer cells to infiltrate adjacent stroma and metastasize to distant sites. These phenotypic changes appear to be induced by several miRNAs, such as *let-7, miR-10, miR-34, miR-200,* and *miR-205* [139]. In BC, *miR-155* and *miR-21*, described as oncomiRs, are implicated in EMT, cell migration, and invasion control. A well-known target of *miR-21* is PTEN, a tumor suppressor, which negatively regulates the PI3K pathway [133, 140]. Growing evidence suggests that BC cell plasticity, necessary for the spread of a tumor, arises because of partial reactivation of EMT in a mature cancer cell in order to give the cell pluripotency and a stem-like phenotype.

All the discussed miRNAs are summarized in Table 2.

6. Potential of miRNAs as BC biomarkers

BC is a heterogeneous disease with several morphological appearances, molecular features, behaviors, and response to therapy [143, 144]. Therapeutic management of BC is based on the availability of strong diagnostic, prognostic, and predictive factors to guide the decision and the choice of different treatment options [145-147].

The current *in vivo* diagnostic tools for BC, e.g., mammography and ultrasound, are used for the detection of early-stage BC. However, several technical limitations exist for these techniques, such as breast density or calcification detection. Other imaging modalities, e.g., magnetic resonance imaging (MRI), have been proposed as complementary diagnostic modalities, with limited sensitivity.

Table 2. Examples of miRNAs involved in CSC phenotype, and EMT process. This table focuses on few examples of miRNAs described in the text, with a particular attention on their function and the type of cancer where they have been found.

miRNA annotation	Function	Tumor	Ref.
let-7	Regulator of self-renewal, cell proliferation and EMT	Lung, BC	[124]
miR-34, miR-146a	Symmetric and asymmetric division of CSCs	colon	[132]
miR-34,	Cell cycle control, invasion capacity and metastatic potential	BC	[133, 134]
let-7, miR-10, miR-34, miR-200, miR-205; miR-30	Stemness and EMT regulation	BC	[139, 141, 142]
miR-155, miR-21	EMT, cell migration and invasion control	BC	[133, 140]

Some proteins have been associated with BC by the analysis of expression levels of specific mRNAs, e.g., carcinoembryonic antigen (CEA) and CA-125 [148]. For BC diagnosis and prognosis, several mRNA-based genetic tests are currently available, such as the PAM50 assay (based on the NanoString technology), MammaTyper assay (based on the qRT-PCR technology), MammaPrint test (based on the microarray technology), Oncotype DX test (based on the qRT-PCR technology), Endopredict (based on the qRT-PCR technology), and Genomic Grade Index (based on the microarray technology) [149]. Use of independent cores for gene expression testing in BC, coming from different gene signatures, may be a successful strategy to overcome tumor heterogeneity and sampling error.

Although direct measurements of tissue gene biomarkers have greatly improved BC diagnosis, the invasive and unpleasant nature of the diagnostic procedures limits their application. Isolation and subsequent characterization of circulating miRNAs provide the opportunity to bypass the problems associated with tissue biopsy, which is required as per the currently available genetic tests. In fact, circulating miRNAs are small molecules, found in body fluids (blood, plasma, serum, saliva, urine…). Being important regulators of gene expression and being dysregulated in several types of cancer diseases [150], circulating miRNAs have become interesting in new cancer biomarker research. They have been found to be stably and specifically expressed in mammary tissues and in body fluids when the disease is ongoing [151, 152]. These features enable them to respond to the current clinical needs, allowing them to be used as easy, affordable, and clinically accessible molecular biomarkers in the retrospective analysis of large tissue collections and for diagnosis, prognosis, and prediction of therapeutic outcomes in BC.

6a. miRNAs dysregulated in BC

Several studies have looked at possible specific miRNAs dysregulated in BC with a diagnostic purpose [153, 154]. Dysregulated miRNAs could be divided into 2 groups, being either upregulated or downregulated (Table 3).

Increased expression of *miR-21* has been found *in vitro* in human BC cell lines and tissues, playing a key role in all phases of BC pathogenesis [141, 155], although it also appears to be able to monitor early BC onset [156]. *miR-21* activity controls cell proliferation, G2/M check point, and metastasis diffusion [157-159] and the expression of many anti-oncogenes, including TPM1, programmed cell death 4, maspin, and Bcl-2, to support the metastasis and hyperplasia of BC cells [160].

Several other miRNAs have been validated to be overexpressed in BC; these include the *miR-221/222* cluster [161], *miR-9*, *miR10b*, *miR-29a*, *miR-96*, *miR-146a*, *miR-181*, *miR-373*, *miR-375*, *miR-520c*, and *miR589* [162], highlighting their potential use for BC diagnosis, prognosis, and therapeutic studies [80, 137, 163-165].

Some upregulated miRNAs could cooperate in controlling a network of functional genes to help tumor development or metastasis. Figure 5 shows examples of miRNA regulatory networks in BC that promote metastasis through their ability to coordinately target multiple genes [166]. Ma et al. [167] proved the role of *miR-10b* as a driver of metastasis: *miR-10b,* under the control of the TWIST transcription factor, binds *HOXD10* gene, enhancing cell migration and invasion. HOXD10, in turn, inhibits the Ras homolog gene family, member C (RHOC) protein, favoring metastatic diffusion of the tumor (Figure 5A). The *miR-10b* locus also encodes for *miR-10b*/miR10b-3p*. *miR-10b**, although considered functionally irrelevant, was very recently demonstrated to be important for BC insurgence and development [168]. Hence, if *miR-10b-5p* upregulation leads to the induction of ECM remodeling factors for metastatic invasion, *miR-10b-3p* downregulation is involved in primary BC onset and development, as its overexpression inhibits the proliferation of BC cell lines by targeting cell cycle regulator proteins (BUB1, PLK1, and CCNA2) [168, 169] (Figure 5A).

Among the downregulated miRNAs, *miR-30a*, *miR-31*, *miR34*, *miR-93*, *miR-125*, *miR-126*, *mR-146a*, *miR-195*, *miR-200*, *miR-205*, *miR-206*, *miR-503*, and *let-7* [170-174] have been shown to have a role in BC pathogenesis through the loss of their tumor suppressor properties. Overall, the main mechanisms affected by downregulated miRNAs are cell cycle, proliferation, and metastasis diffusion. Among all, the aforementioned members of the *let-7* family are particularly relevant for BC development, as they function physiologically as tumor suppressors and are often inactivated in cancer [175, 176]. *let-7* miRNAs have been demonstrated to regulate multiple oncogenes, such as RAS, high-mobility group AT-hook 2 (HMGA2), c-Myc, and caspase-3 [177-180], and several genes involved in the maintenance of stem cell phenotype [181]. Thus, downregulation of *let-7* family members could be one of the key events in the initiation of cancer owing to the acquisition of stem cell-like properties [182]. Members of the *let-7* family are among the more reproduced biomarkers identified in the *in silico* research of a miRNA BC signature. In fact, the *let-7* family is present in several miRNA expression signatures of BC tissues [183, 184] and also in other types of tumor [183, 185, 186]. In our recent

publication, we proposed *let-7c* as a possible biomarker of a 4-miRNA signature, capable of distinguishing between grade 1 and grade 3 BC samples [21].

miR-92a is another possibly downregulated miRNA. This miRNA belongs to the *miR-17-92* family, which can promote tumor proliferation by controlling the PI3K/Akt/mTOR pathway [187]. Moreover, this miRNA has Bcl-2 interacting mediator of cell death (Bim) and p53 proteins as targets, thus inhibiting tumor cell apoptosis and cell cycle arrest and promoting tumorigenesis [98, 188, 189]. In addition, it is involved in promoting tumor invasion and metastasis by modulating the TGF-β signaling pathway [190, 191].

Another downregulated miRNA, *miR-206*, has been found to be underexpressed in estrogen receptor (ER)α-positive BC, both in patient samples and BC cell lines [192, 193], and in lymph node metastatic BC [194, 195]. With regard to its functions, it has been recently demonstrated to regulate the 3′ UTR of cyclin D1, inducing G1 arrest and a decrease in cell proliferation in BC cells [196], suggesting a potential role as a tumor suppressor. It has been shown that *miR-206* regulates ERα via interaction with its 3′ UTR [193], demonstrating a specific role in most aggressive types of BC.

Other downregulated miRNAs, typical of BC tissues, are a group of miRNAs usually expressed in stem cells. This group includes the *miR-200* family [197], *miR-15/16*, *miR-103/107*, *miR-128b*, *miR-145*, and *miR-335* [137]. All these miRNAs are downregulated in CSCs, targeting common genes (Bmi1 and Suz12 component, Zeb1/2, and Klf4), all belonging to a regulatory circuit that sustains the breast CSC state [137].

6b. miRNAs as biomarkers of diagnosis, prognosis, and therapy prediction in BC

Several attempts have been made to identify affordable BC signatures for diagnosis, prognosis, and prediction of the therapeutic response (Table 4).

With respect to diagnosis, Iorio et al. [164] identified a 13-miRNA signature that could differentiate BC from normal breast tissues with 100% accuracy. Blenkiron et al. [202] identified 133 miRNAs that displayed aberrant expression levels in breast tumor tissues compared with normal breast tissues. Despite the identification of miRNA with aberrant expression in BC tissues, there remain discrepancies among the different reported miRNA signatures. This is probably because of the intrinsic heterogeneity in BC and because of clinicopathological variables such as the tumor stage, vascular invasion, proliferation index, and expression of HER2, ER, or progesterone receptor (PR). Thus, an attempt has been made to develop a miRNA signature that reflects the histopathological features of the tumor. At the simplest level, BC comprises 3 different histological subtypes: hormone receptor-positive (ER^+, PR^+) tumors, which cover approximately 60%–70% of diagnosed BCs; $HER2^+$ tumors, which cover 15%–20% of diagnosed BCs; and triple-negative (ER^-, PR^-, $HER2^-$) tumors [66]. Genomic mRNA profiling has subdivided BC into 4 different classes: luminal A (ER^+ and low grade), luminal B (ER^+ and high grade), $HER2^+$, and basal like (mainly triple negative) [202].

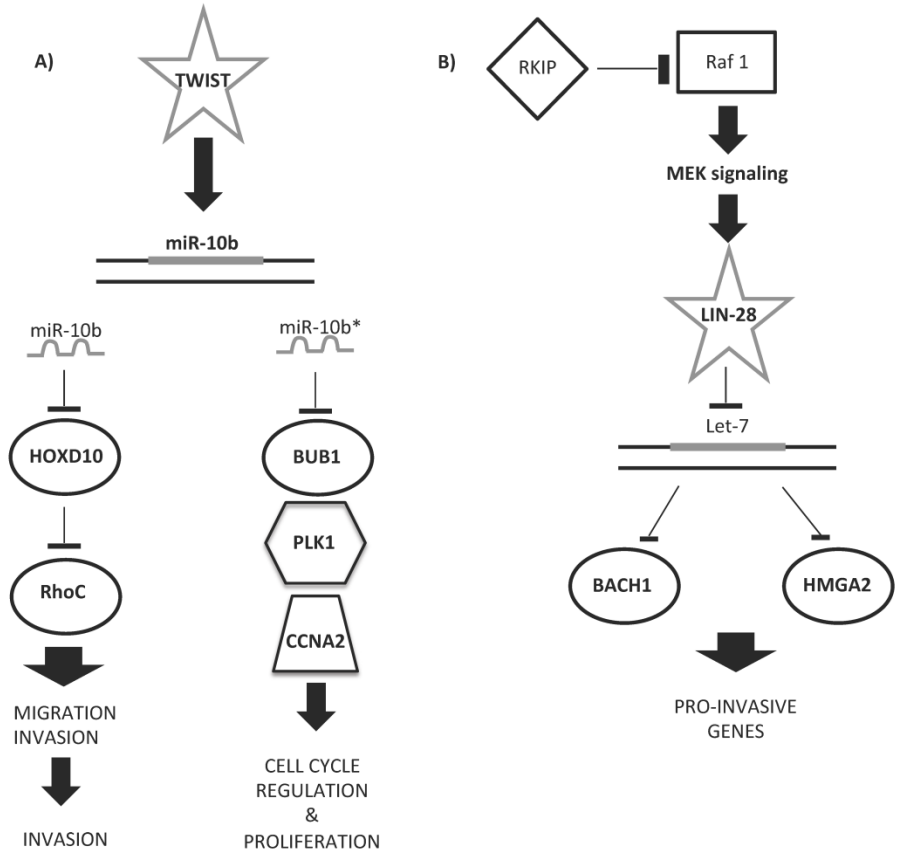

Figure 5: Examples of miRNA regulatory networks in BC that promote metastasis. A) Two examples of the role of *miR-10b*/*10b** in the regulation of either migration and invasion (left side) or cell cycle and proliferation (right side) processes. B) Example of *let-7* regulatory role in the pro-invasive gene network control.

Table 3. BC deregulated miRNAs: an overview.

	miRNA annotations	Samples type	Ref.
Up-regulated miRNAs	*miR-21*	BC cell lines	[141, 155-157, 163]
	miR-221/222 cluster	BC cell lines	[161]
	miR-9, *miR10b*, *miR-29a*, *miR-96*, *miR-146a*, *miR-181*, *miR-373*, *miR-375*, *miR-520c*, *miR589*	BC cell lines	[162-165, 167, 198, 199]
	miR-10b	BC cell lines	[167]
	miR-155	BC cell lines	[158, 163, 200]
	miR-210	BC cell lines	[197]
Down-regulated miRNAs	*miR-30*, *miR-31*, *miR-34*, *miR-93*, *miR-126*, *miR-146a*, *miR-195*, *miR-205*, *miR-206*, *miR-503*	BC cell line; TNBC cell lines	[163, 170-175, 201]
	let-7 family	BC cell lines	[163, 175, 182]
	miR-92a cluster	BC cell lines; TNBC cell lines	[187-191]
	miR-200 family	BC cell lines	[163, 197]
	miR-15/16 cluster, *miR-103/107*, *miR-145*, *miR-335*, *miR-128b*	BC cell lines	[137]
	*miR-10b**	BC cell lines and xenografted tumor	[168, 169]

TNBC= triple negative BC.

Table 4. Circulating and non-circulating miRNAs as BC biomarkers. All the reported miRNAs have been validated on BC patients. For each miRNA, we indicated whether they have a role in diagnosis, prognosis or in prediction of therapy response in BC. For all groups we indicated the biological samples used for the validation, the validation assay, the cohort of data, the main results and the references. This table focused on few examples of single miRNA or miRNA signatures described in the text. GGI=gene expression grade index: TAM=tamoxifen; H= herceptin; N= normal tissue; T= tumor tissues; TNBC= triple negative breast cancer.

Type of miRNAs	miRNA annotation	Role	Biological Samples	Technique/ cohort	Results	Ref.
Non-circulating miRNAs	*13 miRNAs (miR-9-1*, ***miR-10b***, ***miR-21***, *miR-34*, *miR-29b/102*, *miR126*, ***miR125****a/b1/b2*, *miR-140as*, ***miR-145***, ***miR-155***, *miR-194*, *miR-204*, *miR-213)*	Diagnosis	Tissue	Microarray and northern blot/ 76 BC vs 10 N	4/13 are downregulated (**5 miRNAs** are the most constantly deregulated in BC)	[164]
	133 miRNAs	Diagnosis	Tissue	Microarray / 99 BC vs 5 N and 33 BC cell lines	31 miRNAs are associated with tumor subtype or clinical pathological fators	[202]
	15 miRNAs *miR-342*, *miR-299*, *miR-217*, *miR-190*, *miR-135b*, *miR-218.;* *miR-520g*, *miR-377*, *miR-527-518a*, *miR-520f-520c*; *miR-520d*, *miR-181c*, *miR-302c*, *miR-376b*, *miR-30e*	Diagnosis	Tissue	Microarray / 95 BC vs 17 N	ER+; PR+; HER2/*neu+:*	[203]
	6 BC-miRNAs signature *miR-21* *miR-17-5p* *miR-29b-2* *miR-146* *miR-155* *miR-181b-1*	Diagnosis	Tissue	Microarray/ 363 T vs 177 N	31% of the total miRNAs varied among T and N tissues; they identified the most commontly altered miRNAs in solid tumors	[184]
	miR-7, *miR-128a*, *miR-210*, and *miR-516–3p; miR-210*	Diagnosis	Tissue	TaqMan / 185 ER+ vs 114 ER- BC	4-miRNA signature associated with tumor aggressiveness in ER+ BC and miR-210 associated with early relapse in ER-	[204]
	let-7c, miR320dmiR567, miR139-5p	Diagnosis	Tissue	Microarray / 42 BC G1 vs 42 BC G3	4 miRNA signature	[21]
	let-7a *miR-335*	Diagnosis and prognosis	Tissue	TaqMan/ 60 BC vs 60 N	Both miRNAs are decreased in BRCA mutant; miR-335 could be used as prognostic marker	[66]
	miR-155, *miR-493*, *miR-30e*, *miR-27a*	Diagnosis and prognosis	Tissue	Microarray/ 80 high risk vs 80 low risk	2 upregulated, 'protective' miRNAs (miR-155, miR-493); 2 downregulated risk-associated miRNAs (miR-30e , miR-27a)	[205]
	miR-210 *miR-148a*	Prognosis	Tissue	qRT-PCR/ 89 ER+ BC +TAM vs 56 N	miR-210 and miR-148a are associated with relapse free survival;	[206]
	miR-190b *miR-339-5p* *miR-520c-3p /g/h* *miR-139-3p* *miR-204 miR-502-5p* *miR-365 miR-363* *miR-7*	Prediction of therapy response (TAM)	Tissue	Microarray/ 26 patients with recurrence vs 26 patients without recurrence	miR-7 correlates with tumor grade	[212]
	miR-30c *miR-422a* *miR-30a-3p* *miR-187*	Prediction of therapy response (TAM)	Tissue	qRT-PCR/ 38 ER+ BC vs 15 BC+TAM	Higher miR-30a-3p, miR-30c, and miR-182 are associated with treatment benefits	[213]

	miR-182					
	miR-21, miR-181b, miR-26a/26b, miR-27b miR-23b, let-7 family, miR-125a-5p/b-5p	Prediction of therapy response (TAM)	Cell lines and tissue	qRT-PCR/ i) BC cell lines ± ER activation ii) 15 ER+ BC + exemestane and TAM	All miRNAs are upregulated upon anti-estrogen treatment	[214]
	miR-26a, miR-30b, let-7 family, miR-125a/b	Prediction of therapy response (H)	Tissue	Microarray/ 83 BC + H vs adiacent stromal microdissected cells	With SVM technique, they developed a 35 miRNA signature for H treatment response	[216]
Circulating miRNAs	*miR-155*	Diagnosis	Serum	qRT-PCR/ 89 BC vs 29 N	miR-155 is increased, both in primary and metastatic BC	[208]
	miR-195	Diagnosis	Blood	qRT-PCR/ 83 BC patients vs 44 N	miR-195 is increased +19.25 fold	[209]
	miR-29a miR-21	Diagnosis and prognosis	Serum	qRT-PCR/ 20 BC sera vs 20 N	both miRNAs are increased; miR29a correlates with tumor stage	[210]
	miR-16 miR-25 miR-222 miR-324-3 p	Diagnosis and prognosis	Serum	TaqMan / 48 BC vs 48 N	All are increased in high risk patients	[211]
	let-7 miR-21 miR-202	Diagnosis and prognosis	Blood and serum	RT-PCR/ 136 BC vs 60 non BC	All miRNAs are increased in BC patients;	[224]
	miR-18b miR-103 miR-107 miR-652	Diagnosis, prognosis	Serum	RT-PCR/ 33 primary TNBC vs 33 N	4 miRNA signature predict tumor relapse and overall survival	[225]
	miR-210	Prediction of therapy response (H)	Plasma	TaqMan / 18 BC+ H vs 11 not responding BC	miR-210 is higher in patients with residual BC (+2 fold)	[222]
	miR-155	Prediction of therapy response (taxane)	Serum	RT-PCR/ 103 BC+taxane vs 55 N	miR-155 expression correlates with the treatment course	[223]

Blenkiron et al. [202] tried to associate a miRNA profile with each of these genomic classes. Among the 309 miRNAs identified in 93 BCs, 9 miRNAs (*miR-15b*, *miR-99a*, *miR-100*, *miR-103, miR-107, miR-126**, *miR-130a*, *miR-136*, and *miR-146b)* could discriminate luminal A from luminal B BC [202].

Subsequently, Lowery et al. [203] identified a 15-miRNA predictive signature corresponding to the expression of ER (*miR-135b*, *miR-190*, *miR-217*, *miR-218*, *miR-299*, and *miR-342)*, PR (*miR-377*, *miR-520f, miR-520g*, and *miR-527-518a,*), and the HER2 receptor (*miR-30e, miR-181c*, *miR-320c*, *miR-376b,* and *miR-520d*). The same approach was used by Volinia et al. [184], corresponding to the identification of a 17-miRNA signature for the status of ER (*miR-30d* and *miR-30e*), PR (*miR-19a*, *miR-29c*, *miR-30a-5p*, and *miR-106b*), HER2$^+$ (*let-7f*, *let-7g*, *miR-10b*, *miR-107*, *miR-126*, *miR-154*, and *miR-159*), and ER/PR (*miR-25, miR-142-5p*, *miR-200a*, and *miR-205*,).

Finally, Foekens et al. [204] described a subset of miRNAs significantly associated with an ER$^+$ luminal signature, identifying particularly 4 miRNAs associated with BC aggressiveness. The discrepancies among the different miRNA signature studies could result from the fact that miRNAs identified in each study were not examined in the others, besides other variables such as clinicopathological parameters of the tumor (tumor size, grade, etc.) or the use of different detection platforms (RT-PCR, next generation sequencing, etc.).

Hence, in our recent publication, starting from public BC databases containing gene expression profiles, copy number information, and miRNA profiles, we have described new 4 miRNA-based signatures, identifying a small group of miRNAs typical of BC, which could distinguish BC with different grades [21].

Several other small signatures or single miRNAs have been proposed with a diagnostic or prognostic aim [66, 205, 206].

All the described miRNAs are summarized in Table 4, section "Non-circulating miRNAs."

The observation that miRNAs could be secreted by a solid tumor into the surrounding environment and that they are stable in body fluids make miRNAs promising targets easily found in blood, plasma, and serum [207]. Some attempts have been made to identify single circulating miRNA or small circulating miRNA signatures with a diagnostic or prognostic purpose (Table 4, section "Circulating miRNAs"). Few examples of single circulating miRNAs proposed as diagnostic or prognostic tools have been suggested by Roth et al. [208], who found *miR-155* in the serum of patients with BC and not in healthy controls, and by Heneghan et al. [209] who found elevated *miR-195* expression in the blood of only patients with BC. Other miRNAs have been detected in the serum of patients with BC, such as *miR-29a* and *miR-21* [210] or the 4-miRNA signature of Hu et al. (*miR-16*, *miR-25*, *miR-222*, and *miR-324-3p*) [211] (Table 4).

The leading strategy for BC treatment is the use of surgery in combination with or followed by chemotherapy. The most common chemotherapeutics are antracyclines [doxorubicin (DOXO), adriamycin (ADR), and epirubicin], selective ER modulators [ta-

moxifen (TAM)], taxanes (taxol or paclitaxel and docetaxel), 5-fluorouracil (5-FU), and cyclophosphamide. Despite advances in treatment achieved by the combination of some of these compounds, a large number of patients do not respond to chemotherapy. In this context, miRNAs that are able to predict the therapeutic response of a given patient could help clinicians in the choice of the correct therapeutic approach. Some signatures have been developed in search for miRNAs able to predict the therapeutic response of patients with BC [206, 212-214] (Table 4). TAM, one of the main molecules used in BC treatment, is a drug that reduces or eliminates circulating estrogen or blocks the interaction of ER with genomic targets. In some studies, it was evaluated whether TAM could be a successful treatment for ER$^+$ BC. The analyzed population had already developed metastasis prior to the onset of treatment and the benefit of treatment was measured as an objective response according to the REMARK criteria [215].

Other miRNA signatures have been studied for predicting the response of BC to Herceptin (or trastuzumab, H) [216]. This molecule is a recombinant humanized monoclonal antibody against HER2 proteins that blocks the HER2-mediated activation of intracellular kinases and effectors [66]. Although H treatment prolongs survival in adjuvant and metastatic settings, a majority of women with HER2$^+$ metastatic disease will develop resistance to the therapy within a year of treatment. Identification of a miRNA signature that predicts patient risk, disease outcome, and tolerability to H therapy would greatly improve the personalized management of HER2$^+$ BC. A few studies have successfully identified a prognostic miRNA signature for the response of BC tissue samples to H therapy [217]. Combining the results of non-circulating miRNA signatures of BC cell lines treated with TAM or H, in order to identify miRNAs with predictive ability, only the *let-7 family* and *miR-125a-5p/b-5p* emerge as important predictors of therapeutic response [214, 217]. *miR-125*, whose expression correlates with the HER2 status [218], has been found to be significantly downregulated in patients with BC [183, 219]. Experimentally, the overexpression of *miR-125* reduces ERBB2 and ERBB3, decreasing cell motility and the invasiveness of numerous cancers, including BC [219]. The *let-7* regulatory network suppresses metastasis by directly targeting the chromatin-remodeling protein HMGA2 and the transcription factor BACH1 [180, 220] (Figure 5B). Both targets promote the transcription of pro-invasive genes that suppress cell invasion and metastasis to the bone [180, 220].

Almost all publications of circulating miRNA profile and HER2$^+$ BC response to therapy have used BC cell lines to identify single miRNAs or groups of miRNAs whose expression is altered after prolonged H treatment [217, 221]. One miRNA possibly involved in the response to H therapy is *miR-210*, which is present both in tissue and in body fluids of patients with BC [222]. Circulating *miR-210* has been associated with H sensitivity, tumor presence, and lymph node metastasis, suggesting a possible use of *miR-210* to monitor the response of HER2$^+$ BC to H-based therapies [222]. Another miRNA, *miR-155,* has been used to monitor the effect of taxane treatment on BC. Sun et al. observed the decreased expression of *miR-155* in serum after chemotherapy, which reached levels comparable to those of healthy subjects [223].

6c. miRNAs and hallmarks of BC

We have depicted an overview of miRNAs that can already be considered as BC biomarkers. This is outlined in Table 4. We have tried to classify all circulating and non-circulating miRNAs with diagnostic, prognostic, and predictive capacity in relation to their function as described in the literature. In particular, we have related them to altered pathways, the hallmarks of BC [226], generating a daisy-shaped figure (Figure 6) in which each of the petal represents one of the hallmark function altered in BC. The major group of miRNAs (27 miRNAs) affects genes belonging to the proliferation pathway, although some of these miRNAs (*miR-155*, *miR-210*, *miR-21*, and *let7 family*) are also involved in directing the BC invasion and metastatic pathways, controlled by other 24 miRNAs. Resting cell death and apoptosis are the targets of the third larger 14-miRNA group. Five miRNAs are responsible for the control of angiogenesis, whereas 2 miRNAs control genomic instability. *miR-210* is the miRNA with a wider activity, being involved in energy metabolism, angiogenesis, and genomic instability beside the already described role in invasion and proliferation. Other miRNAs, such as *miR-21, miR-27a/b,* and *miR-155*, have been demonstrated to have multiple functions. This feature may be partially explained by the fact that the action of certain miRNAs is dependent upon the cellular model or environmental context in which they have been studied. Only 5 miRNAs have not yet been characterized *in vitro* for their function in BC development (*miR-213*, *miR-299*, *miR-422a, miR-493*, and *miR-527*).

With respect to the clinical use of the depicted miRNAs, the majority of them (33/59) can be used as diagnostic tools; a small number of them (7/59) (indicated in italics in Figure 6) also have prognostic ability (members of the *let-7* family, *miR-27a*, *miR-30*, *miR-148a*, *miR-155*, *miR-210*, and *miR-335*). Some of them (19/59) have the capacity to predict the response of BC to therapy (indicated with * in Figure 6; *let-7*,

miR-7, *miR-21*, *miR-23b*, *miR-26a/b*, *miR-27b*, *miR-30b/c*, *miR-125 a/b*, *miR-139-3p*, *miR-181b*, *miR-182*, *miR-187*, *miR-204*, *miR-210*, *miR-339-5p*, *miR-363*, *miR-365*, *miR-502-5p*, and *miR-520* family); 10/59 (marked in red in Figure 6) are circulating miRNAs, showing different functions in BC, and 2/59 (*miR-155* and *miR-210*) are circulating miRNAs with both diagnostic, prognostic, and predictive role in BC.

7. miRNA for therapeutic use in cancer

Use of miRNAs for the development of new therapeutic strategies is based on 2 approaches: 1) use of miRNAs as drug molecules, based on the synthesis and delivery of specific oligonucleotides, able to increase or decrease miRNA levels in BC or 2) modulation of miRNAs in combination with non-miRNA-based therapies to increase the efficacy of the conventional treatments.

7a. Methods for miRNA modulation

There are 2 main approaches for developing miRNA-based therapies: antagonist and mimic oligonucleotides. MicroRNA antagonists or antagomiRs are generated to inhibit miRNAs that acquire a gain of function in human disease.

The most common strategy to ablate the function of miRNAs is achieved by single-stranded oligonucleotides with miRNA complementary sequences. In contrast, miRNA mimics are used to restore miRNAs that show a loss of function, as in the traditional gene therapy. This approach, also known as miRNA replacement therapy, has attracted much interest as it provides a new opportunity to therapeutically exploit tumor suppressors. The low molecular weight of miRNAs permits the delivery of therapeutic miRNAs as short double-stranded oligonucleotides [227]. To improve the efficiency of miRNA/anti-miRNA delivery *in vivo,* modified miRNA molecules, both miRNA mimics and antagomiRs, with longer half-lives and increased efficiency have been developed, such as anti-miRNA oligonucleotides (AMOs) [228], locked nucleic acid (LNA)-modified oligonucleotides [229], cholesterol-conjugated antagomiRs [230], and the recently developed 2′-O-methoxyethyl-4′-thioRNA (MOE-SRNA) [231].

In recent years, a method has been described to inhibit miRNA function using synthetic mRNAs containing multiple binding sites for a specific miRNA, called miRNA sponges [232, 233]. In bladder cancer cell lines it has been demonstrated that the forced expression of a miRNA sponge designed to inhibit *miR-21* leads to a reduction in tumor aerobic glycolysis, i.e., the ability of the cells to metabolize glucose even under aerobic conditions [234]. miRNA sponges have been validated even in an SUM149-epithelial BC cell xenografted mouse model, where inhibition of Myc-driven *miRNA-9* using a synthetic mRNA containing several *miR-9* binding sites reduced the development of lung metastases [198]. Inhibition of the BC cell proliferation effect has been observed even in another xenografted mouse model implanted with the MDA-MB-231 BC cell line, where a *miR-150* sponge-based inhibition led to a reduction in tumor mass proliferation via increase in the P2X7 receptor [235].

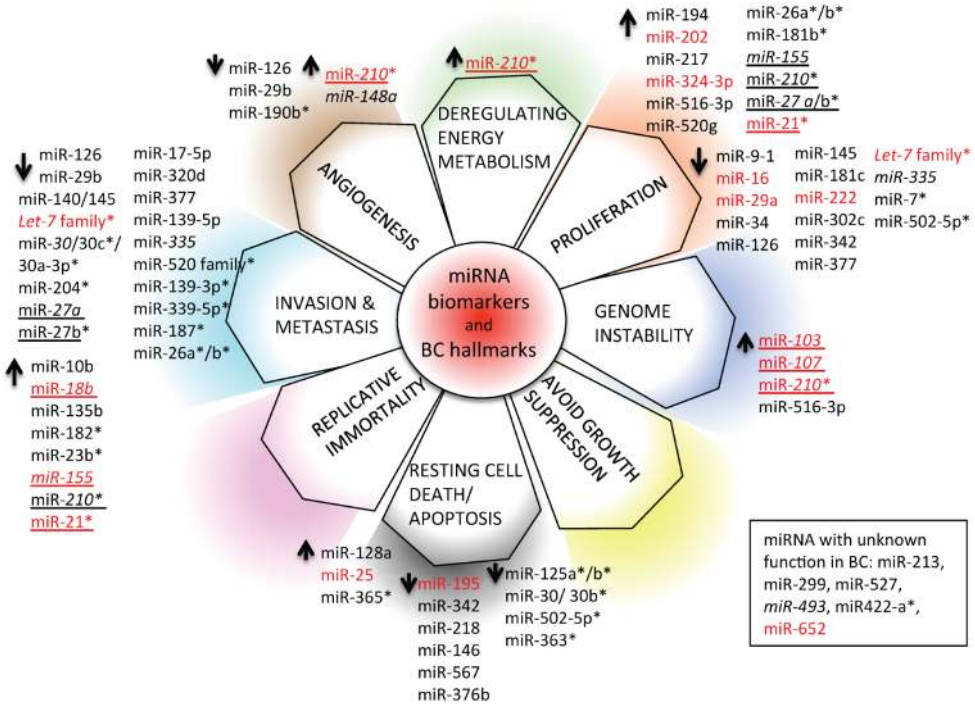

Figure 6: **miRNA biomarkers and BC hallmarks.** miRNAs have a role as diagnostic miRNA, prognostic miRNAs *(italics)*, miRNAs predictive of the BC response to therapy (*), or miRNAs with multiple functions (diagnosis, prognosis, prediction of therapy outcome; underlined). Circulating (red) and non-circulating (black) miRNAs of Table 4 are included.

7b. miRNA-targeted therapies

Use of miRNAs alone in anti-cancer therapy to inhibit BC proliferation and development is still a challenge, although some promising results have already been obtained in both *ex vivo* and *in vivo* experiments. For instance, *miR-145* has been chosen as a target therapy in BC cells because it is usually found to be downregulated in BC [236]. Use of mimic or inhibitor miRNA oligonucleotides has been exploited in *in vivo* experiments. For instance, *miR-21* has been found to be of particular interest in BC because chemically modified anti-miRNA oligonucleotides have already been developed for the *in vivo* treatment of xenografted BC mouse models [110].

To avoid rapid degradation and excretion of miRNAs, study of new delivery systems, which could enhance the stability and the delivery to target tissues, is necessary.

Recently, some studies focused on the potential use of nanomaterials to facilitate the delivery of biomolecules inside tumors. In particular, gold nanoparticles, with their high affinity for biomolecules, reduced cytotoxicity, easy size control, and well-developed surface chemistry, have been modified to increase their complementarity for nucleic acids [237], allowing the effective delivery of silencing RNAs inside cells [238, 239]. The same approach has been used for the delivery of *miR-145* into BC cells [236]. Therefore, gold nanoparticles have been successfully used for the delivery of *miR-145* oligonucleotides inside BC cell lines [240].

The question regarding the effect of the delivery on non-target organs and systemic toxicity of the compound remains unanswered. An obstacle for the use of miRNAs in therapy is the fact that miRNA modulation can affect hundreds of transcripts in different tissues, being potentially capable of shutting down entire pathways. Thus, till date, few companies have used miRNAs to develop a new class of cancer therapeutics. MRX34, a *miR-34a* mimic compound, is probably one of the first miRNA replacement agents to undergo clinical trials. At the time of writing this review, Mirna Therapeutics is recruiting participants to a phase I study of MRX34 (NCT01829971) (http://clinicaltrials.gov/ct2/show/NCT01829971). Similarly, Regulus Therapeutics is developing antago-*miR-221* for hepatocellular carcinoma treatment and antago*miR-10b* for glioblastoma treatment (http://www.regulusrx.com/therapeutic-areas/#Oncology). All these companies suggest that antago-miR oligonucleotides can be easily administered through local or parenteral injection routes with sufficient uptake of the agent to achieve sustained target inhibition in tissues and organs without the need for a formulation. Nevertheless, miRNA features, such as their stability and widespread activity on several targets, lead us to think that before using miRNA in therapies, much work is required to obtain more detailed and comprehensive knowledge about miRNA therapeutic potential, such as miRNA tissue distribution and systemic toxicity.

7c. miRNAs and chemoresistance

miRNAs can potentially be used to increase the response of BC to a therapeutic intervention. As an example, BC has been shown to be chemoresistant when some miRNAs are dysregulated (e.g., *miR-125b*, [239]). miRNA mimic oligonucleotides, which increase the levels of a given miRNA in a BC in which this miRNA is lost, can be used in combination with conventional therapy to obtain an increased benefit for patient outcomes. An example of this approach has been used in the study by Yang et al. [241], where the upregulation of *miR-195*, obtained by mimic oligonucleotides, supplied to ADR-resistant MCF7 BC cell lines increased the sensitivity of the cells to the treatment, leading to apoptosis through downregulation of Raf-1 and Bcl-2. In addition, the combined treatment of a patient with BC using antago-miR oligonucleotides to shut down the increased miRNA expression levels in a specific patient could potentially increase the effect of conventional therapy. This approach has been used, for example, to demonstrate *in vitro* that *miR-21* antisense oligonucleotides could be used in combination with H to kill resistant BC cells in xenografted mouse models [242].

For a more detail review on the role of miRNAs in chemoresistance modulation, see [243].

7d. miRNAs, CSCs, and chemoresistance

The ability of miRNAs to regulate the CSC phenotype and EMT is critical in the management of therapy resistance. In fact, CSCs in tumors are resting populations. The existing common chemoradiotherapy can target only rapidly proliferating cells. Thus, CSCs will escape from being killed and will thus be able to become resistant, to metastasize, or to generate relapses. miRNA modulation is therefore fundamental to increase the response of the tumor to the therapy. It has been demonstrated, for example, that a leukemia cell line resistant to daunorubicin shows increased levels of *miR-21*, the miRNA responsible of the control of EMT, whereas the suppression of this miRNA in the same cell line enhances daunorubicin cytotoxicity [244]. Furthermore, in BC, therapy resistance is clearly influenced by CSC miRNAs. For example, *miR-34a* expression, necessary for the maintenance of CSC, is downregulated in BC ADR-resistant cell lines, and its upregulation leads to increased sensitivity to therapy, both *in vitro* and *in*

vivo [245]. Moreover, a close cross-talk among *miR-200c*, *miR-203,* and a stem cell transcription factor, Bmi1, has been described in BC; in particular, Yin et al. [246] demonstrated that Bmi1, often upregulated in BC and involved in stem cell maintenance, is regulated by *miR-200c* and *miR-203*. Bmi1 expression, and the parallel *miR-200c* and *miR-203* downregulation are accompanied by the reversion of resistance to chemotherapy treatment in different BC cell lines [246].

In all these examples, it is clear that modulation of miRNAs has a critical impact on CSC maintenance, EMT, and, in general, on the response of the disease to therapy. Because CSCs are involved in the relapse of BC, modulation of miRNA in combination with therapy could decrease the possibility of next BC recidivism.

8. Conclusions and future perspective

In this review, we have focused on the recent advances related to miRNAs involved in BC and on their capability to respond to the actual clinical needs for the diagnosis, prognosis, and treatment of patients with BC.

Several lines of evidence have proven that in cancer, including BC, alterations in the levels of miRNAs are not only due to alterations in the miRNA biogenesis mechanisms but also depend on several upstream steps, such as epigenetic control, transcription factors, or mutated protein controls. As a consequence, prolonged aberrant expression of miRNAs, which could act either as tumor suppressors or as oncogenes, may contribute to the onset, progression, and diffusion of BC.

In the recent years, considerable progress has been made to understand the mechanisms responsible for aberrant miRNA expression in BC and several miRNAs or miRNA families have been found as key regulators of BC hallmarks (Figure 6).

Because early diagnosis of BC is essential for better prognosis of patients and because the currently available diagnostic methods show some limitations, miRNAs are emerging as novel diagnostic and prognostic biomarkers for BC. In particular, several miRNAs have shown BC diagnostic potential (i.e., *miR-9, miR-10b, and miR-17-5p*) and other miRNAs have shown a role in BC prognosis (i.e., *miR-148a* and *miR-335*). BC miRNAs have also been proposed as predictors of therapeutic outcomes, because their expression levels enable the prediction of patients' response to specific treatment (i.e., *miR-30c*, *miR-187*, and *miR-339-5p*). A significant number of these miRNAs, found as BC biomarkers, have an important role in the control of BC hallmark functions such as invasion, metastasis, proliferation, resting death, apoptosis, and genome instability.

A few miRNAs have multiple roles in diagnosis, prognosis, and prediction of therapeutic response in BC. Among these miRNAs, some are circulating (e.g., *miR-155* and *miR-210*), and owing to their stability in body fluids, could really become new, easily accessible, affordable, non-invasive, and promising testing tools for the personalized management of patients with BC. Nevertheless, recent evidence suggest that circulating, multiple miRNAs-based profiles have better diagnostic and prognostic performance as well as better sensitivity than individual miRNA assays [225, 247] because the combination of several miRNAs, controlling multiple target genes, can better clarify how each of them contributes to tumor development and better represent the global biological effect of miRNA regulation on the multistep process leading to BC.

However, the full potential of miRNAs should not be exhausted in their use as biomarkers of BC. Future research should be directed to the development and delivery of miRNA-based drugs in BC; in this respect, some miRNAs have already shown promising results (e.g., *miR-9, miR-21, miR34a, miR145, and miR150*). Thus, particular attention should be given to the optimization of miRNA-based drug stability, improvement of miRNA delivery, and control of the off-target effects of miRNA therapeutics. Moreover, to increase the efficacy of currently used non-miRNA treatments for BC (e.g., chemotherapy), great efforts should be directed to the use of such non-miRNA treatments in combination with miRNAs showing a fundamental role in the modulation of the response to treatment (e.g., *miR-21, miR34a, miR195, miR200c,* and *miR203* in combination with chemotherapy).

Abbreviations

BC: breast cancer; miRs or miRNAs: microRNAs; TAM: tamoxifen; H: herceptin or trastuzumab; ADR: adriamycin; CSC: cancer stem cells; EMT: epithelial-mesenchimal-transition; MET: mesenchymal-epithelial-transition; TICs: tumor initiating cells; N: normal tissue; T: tumor tissue; ER: estrogen receptor; PR: progesterone receptor; ERBB2/HER2: the receptor tyrosine-protein kinase erbB2.

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Author contributions

GB and CC wrote the manuscript, and collaborate in figure management and technical editing of the

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Competing interests

The authors have declared that no competing interest exists.

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