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Review

Intracellular and Extracellular MicroRNAs in Breast Cancer

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BACKGROUND: Successful treatment of breast cancer is enhanced by early detection and, if possible, subsequent patient-tailored therapy. Toward this goal, it is essential to identify and understand the most relevant panels of biomarkers, some of which may also have relevance as therapeutic targets.

METHODS: We critically reviewed published literature on microRNAs (miRNAs) as relevant to breast cancer.

SUMMARY: Since the initial recognition of the association of miRNAs with breast cancer in 2005, studies involving cell lines, in vivo models, and clinical specimens have implicated several functions for miRNAs, including suppressing oncogenesis and tumors, promoting or inhibiting metastasis, and increasing sensitivity or resistance to chemotherapy and targeted agents in breast cancer. For example, miR-21 is overexpressed in both male and female breast tumors compared with normal breast tissue and has been associated with advanced stage, lymph node positivity, and reduced survival time. miR-21 knock-down in cell-line models has been associated with increased sensitivity to topotecan and taxol in vitro and the limitation of lung metastasis in vivo. Furthermore, the discovery of extracellular miRNAs (including miR-21), existing either freely or in exosomes in the systemic circulation, has led to the possibility that such molecules may serve as biomarkers for ongoing patient monitoring. Although additional investigations are necessary to fully exploit the use of miRNAs in breast cancer, there is increasing evidence that miRNAs have potential not only to facilitate the determination of diagnosis and prognosis and the prediction of response to treatment, but also to act as therapeutic targets and replacement therapies.

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Over the past few years, breast cancer death rates have shown an overall decrease compared with previous years. According to the American Cancer Society (www.cancer.org), the breast cancer–related death rate in the US continues to drop more than 2% per year, a trend that began in 1990. This trend is largely due to the development and implementation of improved cancer-screening methods and treatment strategies. Despite these steps toward improving survival and reducing mortality rates, breast cancer still remains the leading cause of death among women younger than 85 years *(1)*. Excluding nonmelanoma skin cancers, in many countries breast cancer is the most common cancer diagnosed in women. According to the American Cancer Society, there is a 1 in 8 chance of a woman developing breast cancer at some stage during her lifetime. This disease is not restricted to women, as approximately 1% of all cases occur in men. If breast cancer is detected and treated at an early stage, successful outcomes can be achieved in some patients. Despite this, breast cancer continues to be an immense problem worldwide, with more than 1.3 million cases in women diagnosed annually, resulting in approximately 465 000 deaths per annum *(2)*; this number is predicted to increase in the future *(3)*.

As with many cancers, progress in early breast cancer detection has been inadequate *(4)*, and methods for determining diagnosis and prognosis of breast cancer are still limited to invasive procedures, such as tissue biopsies for histological examination *(5, 6)*. Advances in understanding the cancer cell at the molecular level have enabled development of several targeted therapies that have advanced the treatment of relevant patient subgroups. However, a more extensive range of targeted therapies is urgently needed for treatment of patients in whom the available drugs are unsuitable and for patients who initially respond, but subsequently become resistant to these agents. To achieve such individualized treatment, appropriate targets must be identified, characterized, and validated. It is also important to consider that patients who are unresponsive to administered therapeutic agents are still subject to their adverse effects. Thus, an important focus of current breast cancer research is to increase our understanding of the biology of this disease and to identify panels of biomarkers that may help in early diagnosis and the determination of prognosis and/or the prediction of

treatment response, ultimately contributing to more favorable patient outcomes.

A Brief Introduction to MicroRNAs

THE DISCOVERY OF MicroRNAs

Small RNAs constitute a family of regulatory noncoding RNAs up to 40 nucleotides in length. These small RNAs can induce gene silencing through specific base-pairing with target mRNA molecules. MicroRNAs (miRNAs),[4] a major class of these small RNAs, were initially reported in 1993 in *Caenorhabiditis elegans*. Specifically, *lin-4*, a *C. elegans* heterochronic gene, was found to produce small RNAs (approximately 61-nucleotide precursor; 22-nucleotide mature miRNAs) that were capable of regulating lin-14 mRNA via an antisense RNA–RNA interaction in the 3′ untranslated region (UTR) of lin-14 *(7)*. Since this initial discovery, many miRNAs have been identified in both single-celled and multicellular organisms, including plants and mammalian cells. To date more than 1000 miRNAs have been identified in human tissue (miRBase database, current release http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=hsa), although computational analysis has predicted that many more miRNAs may exist in the vertebrate genome *(8)*.

miRNA BIOGENESIS AND FUNCTIONAL RELEVANCE

Mature miRNAs are small (approximately 18–25 nucleotides long) nonprotein coding RNAs. The first step in miRNA generation begins in the nucleus with a long primary miRNA, produced after miRNA gene transcription by RNA polymerase II *(9, 10)* or, less commonly, by RNA polymerase III *(11)*. The initial step involved in the processing of this primary miRNA is mediated by the microprocessor complex. This complex is comprised of the double-stranded RNA-specific ribonuclease III–type endonuclease, Drosha, with its cofactor DGCR8, a double-stranded RNA-binding protein responsible for recognizing the hairpin loop of the primary miRNA and ensuring correct cleavage by Drosha. This step results in a hairpin-shaped precursor miRNA of approximately 70 nucleotides in length *(12–14)*. This precursor miRNA is then transported across the nuclear envelope to the cytoplasm by the nuclear transport protein, termed exportin 5, where it is further modified by a second RNase enzyme, termed Dicer, which acts in conjunction with a double-stranded RNA-binding protein (TRBP/TARBP2P). In mammalian cells, TRBP recruits argonaute protein (Ago2/EIF2C2); together Dicer, TRBP, and Ago2 form the RNA-induced silencing complex. Cleavage of the precursor miRNA by Dicer produces a mature miRNA/miRNA nucleotide duplex approximately 22 nucleotides in length, 1 strand of which will become the mature miRNA *(13)*. The Ago2-bound mature miRNA subsequently is assembled into effector complexes termed miRNA-containing ribonucleoprotein particles. Within the silencing complex, miRNAs pair to the messages of protein-coding genes, usually through imperfect base pairing within the 3′-UTR. The "seed" region (nucleotides 2–7) at the 5′ end of the miRNA is often sufficient for its specificity and functionality. The effect of an miRNA is generally negative regulation of gene expression, by acting posttranscriptionally. However, miRNAs have also been implicated in regulating mRNA stability (including degradation by accelerating decapping and deadenylation) and compartmentalization.

As we have previously detailed *(10)*, several proposed models exist for the mechanism of translational repression, including miRNAs repressing translation at both preinitiation and postinitiation stages. However, it still remains to be deciphered which of these model mechanisms are cause and consequence of translational repression. miRNAs that affect initiation steps are possibly involved in m^7G cap recognition, because only cap-dependent translation is effected by miRNA action. Argonaute proteins contain structural similarities to cap-binding protein eIF4E, and so it has been suggested that translational repression may occur owing to competition between argonaute proteins and eIF4E for binding to the cap structure. Argonaute proteins are also thought to recruit eIF6, which binds to the large ribosomal subunit, preventing binding of the small subunit, thus inhibiting mRNA translation. Postinitiation mechanisms of repression that affect both cap-dependent and cap-independent translation also exist. Polysome profile experiments indicate that, under conditions of translational repression, target mRNAs are fully loaded with ribosomes, a number of which are engaged in active translation, suggesting that translation initiation and elongation phases are not compromised. Two possible hypotheses have been proposed to explain these findings. The ribosome "drop-off" theory suggests that ribosomes engaged in translation of miRNA-associated mRNAs are prone to terminate translation prematurely. Alternatively, association of active ribosomes with repressed mRNAs may also be explained by the ability of the miRNP complex to recruit proteolytic enzymes to degrade the nascent polypeptide as it emerges from the ribosome. Conflicting evidence exists on the role of proteolytic enzymes in

[4] Nonstandard abbreviations: miRNA, microRNA; UTR, untranslated region; qPCR, quantitative reverse-transcription PCR; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Sp, specificity proteins; VEGF-A, vascular endothelial growth factor A; CYP1B1, cytochrome P4501B1; EMT, epithelial-to-mesenchymal transition; TGF-β, transforming growth factor β; NF-κB, nuclear factor-κB; T+LET R, testosterone-only MCF-7 cells that are resistant to the aromatase inhibitor letrozole.

miRNA function, because targeting of reporter proteins and the use of proteinase inhibitors have generally shown no effect on translational repression.

Induction of mRNA decay is another mechanism by which miRNAs apply their actions. Argonaute proteins, miRNAs, and their repressed target mRNAs have been shown to accumulate in cytoplasmic foci called P-bodies, i.e., cytoplasmic processing bodies in mammalian cells. P-bodies are sites of translational repression and mRNA decay, are rich in factors associated with these processes, and are lacking in ribosomes or any other factors associated with translation initiation. It has been proposed that P-body proteins may participate in the formation of a repressive complex on the target mRNA that could eventually lead to mRNA aggregation into P-bodies. Within P-bodies, miRNA/mRNA-bound Ago protein recruits GW182 protein, which subsequently recruits deadenylase enzyme Ccr4:NOT1, which is followed by mRNA decapping by Dcp1:Dcp2 enzyme. Repressed mRNAs are then degraded by 5′ to 3′ exonuclease activity of Xrn1 (5′-exoribonuclease 1). In addition to facilitating mRNA degradation, P-bodies may function as temporary storage sites for repressed mRNAs. Once protein synthesis has been stimulated, repressed mRNAs may reenter translation.

The relevance of miRNAs rests on their involvement in and subsequent effects on many diverse biological functions. Since the discovery of miRNAs 17 years ago *(7)*, it has been estimated that miRNAs regulate more than 60% of all human protein-coding genes *(15)*. miRNAs have been implicated in the control of a wide range of essential biological activities, including cellular proliferation *(16)*, differentiation *(17)*, and apoptosis *(18)*. Importantly, the association of miRNAs with cancer (see below) has prompted additional functional classification of these short RNAs and their potential relevance in cancer diagnosis, prognosis prediction, and treatment.

The Role of miRNAs in Breast Cancer

The association of miRNAs with tumor biology was first determined on the basis of observed deletions and downregulation of miR-15 and miR-16 in B-cell chronic lymphocytic leukemia *(19)*. More recently, through the application of various technologies, including miRNA arrays, bead-based flow cytometry, and quantitative reverse-transcription PCR (qPCR), it has been possible to propose cancer-specific "miRNA fingerprints" for several other cancer types *(20)*, including breast cancer *(21)*, lung cancer *(22, 23)*, and colon carcinoma *(24)*. These initial findings, which suggest that miRNAs have a role in human cancer, have been further supported by the fact that >50% of miRNA genes are located at chromosomal regions, including fragile sites and regions of deletion and amplification that are genetically altered in human cancers *(21)*.

In one of the initial studies cited above, an investigation of differential miRNA expression between tumor and normal breast tissue was performed by Iorio and colleagues *(21)*. Specifically, miRNA microarray profiling (on chips that included 245 human and mouse miRNAs) of 76 primary breast tumors and 10 normal breast samples identified 29 significantly differentially expressed miRNAs. Some miRNAs (e.g., miR-21 and miR-155) were upregulated in breast cancer, whereas others (e.g., miR-10b and miR-145) were downregulated, suggesting that miRNAs may act as oncogenes and tumor-suppressor genes, respectively. Furthermore, considering other clinical features, including lymph node status; estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2, also termed erbB2) status; proliferative index; and presence or absence of vascular invasion, small numbers of differentially expressed ($P < 0.05$) miRNAs were identified in most comparisons performed. (Exceptions to this were found in comparisons of HER2-positive vs HER2-negative tumors and lobular vs ductal carcinomas. Specifically, analyses of lobular compared to ductal and, similarly, HER2-positive compared to HER2-negative tumors did not reveal any differentially expressed miRNAs.) For the most part, however, this differential expression suggests that miRNAs may have a role in defining molecular and pathological profiles of breast cancer. Building on this knowledge and integrating analysis of miRNA, mRNA, and genomic changes in primary breast tumors, considering basal-like, HER2-positive, luminal A, luminal B, and normal-like subtypes, investigators have identified miRNAs that could be used to classify basal vs luminal subtypes in an independent (albeit small; n = 5 cases) data set. Interestingly, the expression levels of Dicer and Ago2 were found to correlate with tumor subtypes, with Ago2 levels increased and Dicer levels decreased in the more aggressive basal-like HER2-positive and luminal B-type tumors *(25)*.

Given that approximately 1% of breast cancer occurs in men, 319 miRNAs were analyzed in 9 male formalin-fixed paraffin-embedded primary breast tumors and control specimens by using fluorescence-labeled bead technology. qPCR validation was performed on an independent cohort of 12 tumors. Interestingly, as for tumors from women, miR-21 was among the miRNAs most prominently upregulated in cancer (as well as miR-519d, miR-183, miR-197, and miR-493–5p), whereas miR-145 and miR-497 were most prominently downregulated *(26)*.

The differential expression of miRNAs in breast tumors thus far identified in tumor tissue from both female and male breast cancer patients compared with normal breast tissue, and the indication of associations between miRNAs and tumor subtypes, suggest a potential role for such molecules in diagnostic biomarker panels. In addition to this work, further independent studies of miRNAs in these cohorts are now necessary to identify and validate the most relevant miRNAs for this purpose.

miRNAs AS ONCOMIRS

Oncogene miRNAs, now commonly termed oncomirs, may act by hindering the expression of tumor-suppressor genes and/or genes responsible for apoptosis and differentiation *(27, 28)*. Investigations to identify functional oncogenic role(s) for miRNAs have, for the most part, involved their individual manipulation in breast cancer cell-line models and subsequent assessment of associated phenotypic changes. Below we have summarized examples of miRNAs that have apparent oncogenic activity in breast cancer. Although space limitations do not allow the inclusion here of a comprehensive description of all possible oncomirs, what follows strongly supports the proposal of miRNAs as oncogenes contributing to this form of cancer.

miR-21. As outlined above, miRNA-21 exemplifies an miRNA that is implicated as an oncomir in both female and male breast cancer. In an effort to investigate whether miR-21 may have relevance not only as a potential biomarker, but also as a functional target, MCF-7 cells were transfected with anti–miR-21 oligonucleotides. This resulted in a reduction of in vitro cell growth and in vivo tumor growth in a xenograft mouse model. The reduced cellular proliferation was accompanied by increased apoptosis, associated with reduced levels of Bcl-2 antiapoptotic protein *(29)*. miR-21 expression has subsequently been shown to influence several other relevant targets, including the programmed cell death 4 (neoplastic transformation inhibitor) (*PDCD4*)[5] *(30, 31)*, tropomyosin 1 (alpha) (*TPM1*) *(32)*, phosphatase and tensin homolog (*PTEN*) *(33)*, and TIMP metallopeptidase inhibitor 3 (*TIMP3*) genes in breast cancer *(34)* (see Fig. 1).

miR-155. With the use of libraries of synthetic miRNAs to probe the TRAIL (tumor necrosis factor–related apoptosis-inducing ligand)-induced apoptosis pathway in MDA-MB-453, introduction of miR-155 (as well as of let-7c, miR-10a, miR-144, and miR-193) was found to be a potent suppressor of apoptosis, putatively through its effects on caspase 3 *(35)*. The tumor-suppressor gene suppressor of cytokine signaling 1 (*SOCS1*) has recently been identified as an miR-155 target. miR-155 overexpression in breast cancer apparently leads to constitutive activation of STAT3 (signal transducer and activator of transcription 3) through the JAK (Janus kinase) pathway, as well as stimulation of breast cancer cells by interferon γ, interleukin 6 and lipopolysaccharide. These findings suggest a role for miR-155 as a bridge between inflammation and breast cancer *(36)*.

miR-27A, miR-96, and miR-182. miR-27a has also been proposed as an oncomir. Some studies leading to this conclusion have involved analysis of a family of general transcription factors termed specificity proteins (Sp). The Sp family includes 4 members, Sp1, Sp2, Sp3, and Sp4, which act through GC-boxes and related motifs. Angiogenic and proliferative characteristics of breast cancer cells have been associated with overexpression of Sp *(37)*, whereas the zinc finger and BTB domain containing 10 (*ZBTB10*) gene is regarded as a suppressor of Sp. Transfection of MDA-MB-231 cells with anti–miR-27a has been associated with increased ZBTB10 mRNA expression; reduced expression of Sp1, Sp3, and Sp4 at mRNA and protein levels; and reduced Sp-dependent survival and angiogenic genes, findings that support the proposed oncogenic properties of miR-27a *(38)*. In further support of such a role, exposure of SKBR3 cells to a proapoptotic dose of a histone-deacetylase inhibitor, LAQ824, resulted in downregulation of miR-27a levels *(39)*.

Specific and functional target sites for miR-27a, miR-96, and miR-182 exist in the 3′-UTR of the mRNA that encodes the putative tumor suppressor transcription factor FOXO1, which is downregulated in breast tumor tissue compared with normal breast tissue *(40)*. High levels of expression of these miRNAs were detected in MCF-7 cells, in which a very low level of FOXO1 protein is endogenously expressed. The subsequent use of antisense inhibitors to these 3 miRNAs induced an increase in FOXO1 expression and a corresponding decrease in cell number, thus supporting proposed oncogenic capabilities of these miRNAs *(40)*.

Overall, as outlined above, the search to discover miRNAs implicated in oncogenesis has been fruitful. Although we do not propose that those summarized above constitute a comprehensive list of all oncomirs associated with breast cancer, these examples give an

[5] Genes: *PDCD4*, programmed cell death 4 (neoplastic transformation inhibitor); *TPM1*, tropomyosin 1 (alpha); *PTEN*, phosphatase and tensin homolog; *TIMP3*, TIMP metallopeptidase inhibitor 3; *SOCS1*, suppressor of cytokine signaling 1; *ZBTB10*, zinc finger and BTB domain containing 10; *RHOC*, ras homolog gene family, member C; *CD44*, CD44 molecule (Indian blood group);*BRMS1*, breast cancer metastasis suppressor 1; *SOX4*, SRY (sex determining region Y)-box 4; *TNC*, tenascin C; *CDC42*, cell division cycle 42 (GTP binding protein, 25kDa); *CXCR4*, chemokine (C-X-C motif) receptor 4; *CYP19A1*, cytochrome P450, family 19, subfamily A, polypeptide 1.

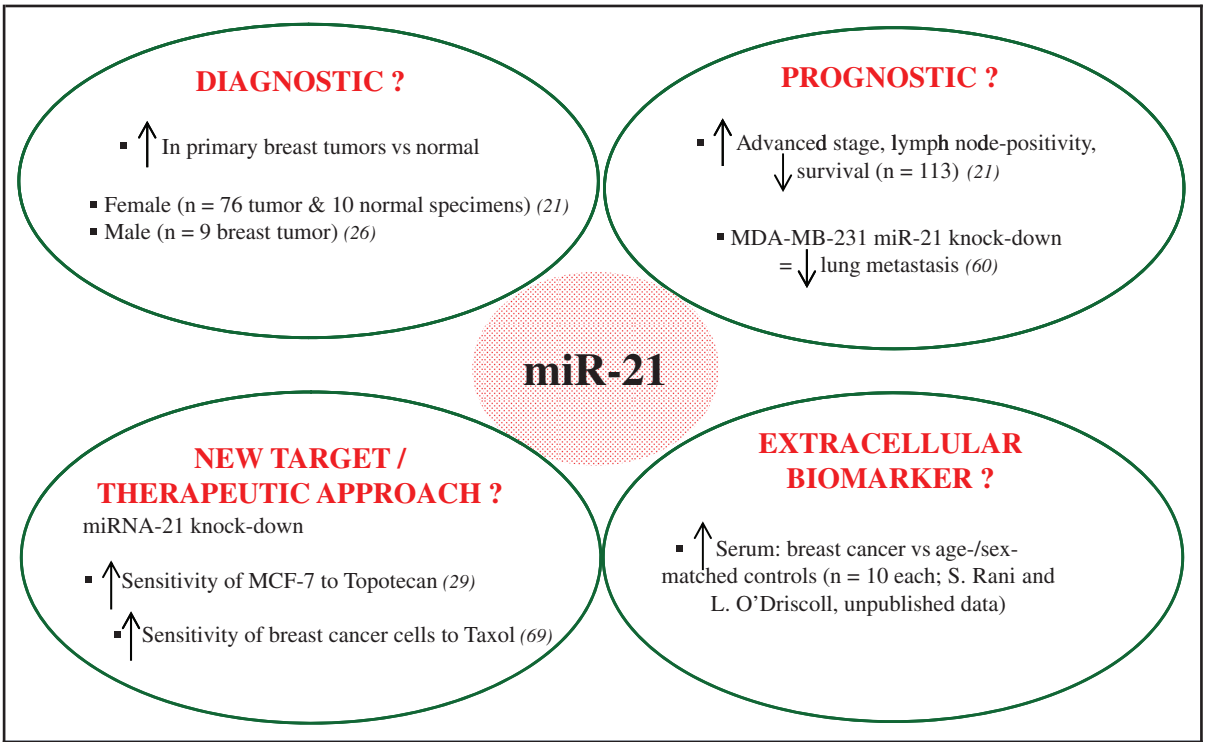

Fig. 1. miR-21 is an example miRNA that shows potential as an intracellular and extracellular biomarker and as a therapeutic target.

Analysis of primary breast tumor and normal specimens support miR-21's role as a diagnostic biomarker. Additional studies in tissue specimens and functional analysis in a cell line model suggest that miR-21 is associated with spread of cancer and poor outcome. Targeted reduction of miR-21 expression in breast cancer cell lines is associated with increased sensitivity to chemotherapy. These findings suggest that treatment regimens that combine anti–miR-21 with classical anticancer agents may aid in overcoming drug resistance and in optimizing treatment. Our pilot studies suggest that miR-21 has relevance as a serum biomarker for breast cancer. References cited in the image: Iorio et al. *(21)*, Lehmann et al. *(26)*, Si et al. *(29)*, Huang et al. *(60)*, Mei et al. *(69)*.

indication of the relevance of miRNAs in this setting. Furthermore, recent advances in bioinformatics have led to the prediction of numerous genes that are potentially regulated by these miRNAs. More comprehensive functional validation of miRNAs is now needed, because many miRNAs have been implicated as oncomirs or as having oncogenic potential based on results of relatively small independent breast cancer studies, but functional characterization and assigning direct role(s) has, as yet, been limited (see examples: Table 1).

miRNAs AS TUMOR SUPPRESSORS

In contrast to oncomirs, if the expression of an miRNA is lowered in cancer cells compared to normal cells, it is regarded as a tumor suppressor (oncosuppressor). Such miRNAs are associated with tumor suppressive activity, because they operate by inhibiting genes that promote tumorigenesis (oncogenes) and control cellular differentiation and/or apoptosis *(27)*. Accordingly, the dysfunction of an oncosuppressor may ultimately lead to the development of malignant cells *(41)*. Here we summarize examples of such miRNAs, which support their role as tumor suppressors in breast cancer.

miR-125A/B. In a study of 20 primary breast cancer biopsy samples, including HER2-positive/ER-negative (n = 9), HER2-positive/ER-positive (n = 4), and HER2-negative/ER-positive (n = 7) samples, levels of both miR-125a and miR-125b were found to be downregulated in HER2-overexpressing tumors *(42)*. Furthermore, miR-125a and miR-125b have both been shown to have tumor suppressive activity in breast cancer cells, with the use of a HER2/HER3-expressing SKBR3 cell line as a model *(43)*. Specifically, by introducing a retroviral construct containing miR-125a or miR-125b into SKBR3 cells, expression of HER2 and HER3 was subsequently suppressed at both the mRNA and protein levels, leading to a reduction in anchorage-

Table 1. Oncogenic miRNAs identified as involved in breast cancer and their potential targets.

miRNA	Clinical specimens/animal models/cell lines used	Targets identified	References
miR-21	Xenograft mouse model, MCF-7, BCAP-37, MDA-MB-231, MDA-MB-435	Bcl-2, *PDCD4*, *TPM1*, *TIMP3*	Si et al. *(29)*, Frankel et al. *(30)*, Lu et al. *(31)*, Zhu et al. *(32)*, Meng et al. *(33)*, Song et al. *(34)*
miR-155	Breast cancer tissue, MDA-MB-453, MCF-7, MDA-MB-231, SKBR3, BT-474, xenograft mouse model	Caspase 3, *SOCS1*	Ovcharenko et al. *(35)*, Jiang et al. *(36)*
miR-27a	MDA-MB-231, SKBR3 MCF-7	*ZBTB10*, FOXO1	Mertens-Talcott et al. *(38)*, Scott et al. *(39)*, Guttilla and White *(40)*
miR-96	MCF-7	FOXO1	Guttilla et al. *(40)*
miR-182	MCF-7	FOXO1	Guttilla et al. *(40)*
miR-128a	MCF-7aro, T+LET R	TGFβR1	Masri et al. *(68)*

dependent growth, cell motility and invasiveness, and associated suppression of ERK1/2 and AKT phosphorylation. In a parallel study performed on the nontransformed/nonmalignant and HER2-independent human mammary epithelial cell line MCF10A, this effect was limited. The potential of exploiting miRNAs as a therapeutic approach (discussed later) to suppress oncogene expression was supported by the results of this study. Expression levels of miR-125a have been reported as inversely correlating with the presence of a stress-induced RNA-binding protein, HuR, which itself is upregulated in a range of different cancers. Overexpression of miR-125a in MCF-7 cells was associated with a decrease in HuR protein concentrations, a reduction in cell proliferation and migration, and increased apoptosis, thus supporting a role for miR-125a as a tumor suppressor for breast cancer, with HuR as a direct and functional target *(44)*.

miR-205. Similarly, studies have implicated miR-205 as a tumor suppressor in breast cancer *(45, 46)*. Wu and colleagues *(45)* reported decreased miR-205 expression in breast tumor tissue (n = 19) and breast cancer cell lines (MCF-7 and MDA-MB-231) compared with matched normal breast tissue (n = 19) and MCF-10A cells, respectively. Furthermore, overexpression of miR-205 in MCF-7 cells was associated with reduced cell proliferation, clonogenic survival, and anchorage-independent cell growth with HER3 and vascular endothelial growth factor A (VEGF-A) proposed as its functional targets *(45)*. The suggestion that miR-205 is an oncosuppressor, negatively regulating HER3, is further supported by the reintroduction of miR-205 into SKBR3 cells, which not only inhibits clonogenic potential, but also eradicates HER3-mediated resistance, thus improving response to tyrosine-kinase inhibitors *(46)*.

miR-27b. A role for miR-27b in breast cancer has been suggested via its relationship with cytochrome P4501B1 (CYP1B1). CYP1B1 functions to catalyze the metabolism of certain procarcinogens and 17β-estradiol, which contribute to the growth and development of estrogen-dependent cancers, such as breast cancer *(47)*. When the expression levels of both miR-27b miRNA and CYP1B1 protein in breast tumors and matched normal tissues (n = 24) were evaluated, in most cases an inverse relationship was discovered whereby miR-27b levels were decreased and CYP1B1 levels were increased in cancer. A near-perfect sequence match for miR-27b in CYP1B1's 3′-UTR further supports the proposal that CYP1B1 is posttranscriptionally regulated by miR-27b *(48)*.

miR-17-5p. miR-17-5P (also known as miR-91) has shown widespread complementarity with amplified in breast cancer 1 (AIB1) mRNA, which as coactivator, in turn, has the capacity to augment the transcriptional activity of nuclear receptors such as the ER *(49)*. In studies involving a range of breast cancer cell lines, through miR-17-5p inhibiting translation, AIB1 expression was reportedly downregulated. This, in turn, resulted in reduced ER-induced and ER-independent gene expression, with a subsequent reduction in cell proliferation. Interestingly, insulinlike growth factor 1–mediated anchorage-independent growth of MCF-7 cells was also completely blocked. Tumor-suppressive effects of miR-17-5p were also indicated in a study investigating the relationship between cyclin D1 and miR-17-5p/miR-20a cluster by use of both in vitro (analysis of MCF-7 cells) and in vivo (analysis of cyclin D1-transgenic mice) analyses. Specifically, a novel feedback mechanism was identified through which cyclin D1 mediates miR-17-5p/miR-20a expression, which in turn, restricts the proliferative role of cyclin D1 *(50)*.

OTHER PUTATIVE TUMOR-SUPPRESSOR miRNAs

In addition to those mentioned above, other miRNAs [including miR-206 *(51, 52)* and miR-145 *(53, 54)*]

Table 2. Tumor-suppressive miRNAs identified as involved in breast cancer and their potential targets.

miRNA	Clinical specimens/ animal models/cell lines used	Targets identified	References
miR-125a/b	Breast cancer tissue, SKBR3, MCF-7	HER2/3, HuR	Mattie et al. *(42)*, Scott et al. *(43)*, Guo et al. *(44)*
miR-205	Breast tumor tissue; MCF-7, MDA-MB-231, SKBR3	HER3, VEGF-A	Whu et al. *(45)*, Iorio et al. *(46)*
miR-27b	Breast tumor tissue	CYP1B1	Tsuchiya et al. *(48)*
miR-17–5p	MCF-7	AIB1	Hossain et al. *(49)*
miR-17/20	Transgenic mice, MCF-7	Cyclin D1	Yu et al. *(50)*
miR-206	MCF-7	ER-alpha	Adams et al. *(51)*, Adams et al. *(52)*
miR-145	MCF-7	RTKN, ER-alpha,	Wang et al. *(53)*, Spizzo et al. *(54)*

have shown potential as oncosuppressors in breast cancer (see Table 2). Interestingly, a higher prevalence of miRNAs with apparent tumor-suppressive activity has been reported, compared to those with oncogenic potential. The finding that an miRNA is up- or down-regulated in tumors compared to corresponding normal tissue is generally the initial evidence indicating possible clinical functional relevance. As outlined above, miRNAs have only recently been examined to elucidate more precisely how they may function to promote or suppress tumorigenesis. This line of research is critical to ascertaining whether miRNAs are potential therapeutic targets, as well as clinical biomarkers.

miRNAs Implicated in Breast Cancer Invasion and Metastasis

Current management strategies for breast cancer are focused on early-stage detection, tumor resection, and neoadjuvant or adjuvant treatment with radiation, chemotherapy, and/or new targeted agents (generally a combination of these approaches). Despite advancements in the treatment of this disease, breast cancer still remains a leading cause of cancer death. Metastasis is the primary reason for high cancer death rates. Hence, to successfully curtail this disease, there is an urgent need to define therapeutic agents that could effectively target cancer before it metastasizes. As outlined above, recent research studies have established the presence of aberrant expression of miRNAs with the potential of either promoting or suppressing tumorigenesis in breast cancer compared to normal breast tissue. The possibility that miRNAs specifically contribute to metastasis has only recently been explored. Several miRNAs have now been described as potentially promoting ("metastamirs") or suppressing metastasis (see Table 3).

miR-10b. One of the first miRNAs implicated as playing a role in metastasis, despite some conflicting evidence, was miR-10b. A high level of miR-10b expres-

Table 3. Role identified for miRNAs in breast cancer metastasis and their potential targets.

miRNA	Role in metastasis	Targets identified	Reference
miR-10b	Promoter	*RHOC*	Ma et al. *(55)*
miR-373	Promoter	*CD44*	Huang et al. *(57)*
miR-502c	Promoter	*CD44*	Huang et al. *(57)*
miR-21	Promoter	HER2	Yan et al. *(58)*, Zhu et al. *(59)*, Huang et al. *(60)*
miR-200b/c	Suppressor	ZEB-1	Tryndyak et al. *(62)*
miR-146	Suppressor	NF-κB	Bhaumik et al. *(63)*
miR-335	Suppressor	*SOX4, TNC*	Tavazoie et al. *(65)*
miR-126	Suppressor	—	Tavazoie et al. *(65)*
miR-206	Suppressor	—	Tavazoie et al. *(65)*
miR-224	Suppressor	*CDC42, CXCR4*	Zhu et al. *(66)*
miR-31	Suppressor	—	Valastyan et al. *(67)*

sion was reported in the metastatic breast cancer line MDA-MB-231 compared to MCF-7 (which has little capacity to metastasize). Transduction of miR-10b into nonmetastatic SUM149 cells resulted in increased size and invasiveness of tumors formed in non-SCID (severe combined immunodeficiency) mice, compared with tumors from control SUM149 cells *(55)*. Although miR-10b–transduced SUM149 tumors invaded the stroma, control SUM149 tumors remained within fibrotic capsules. This study also revealed that miR-10b is activated by the transcription factor Twist, which in turn causes an interruption of homeobox D10 mRNA translation, thus ensuring increased expression of ras homolog gene family, member C (*RHOC*), a gene that promotes cell migration and invasion *(55)*. The expression of miR-10b in primary breast carcinomas (n = 23) compared with normal breast tissue correlates with clinical progression. All breast carcinomas from metastasis-free patients showed low levels of miR-10b expression (5 of 5), whereas high levels of miR-10b expression were detected in 50% of metastasis-positive patients (9 of 18). Results of a more recent study of miR-10b, however, were somewhat contradictory, whereby miR-10b expression was found to be lower in tissue from patients without metastasis (n = 114) compared with normal breast tissue (n = 10). In contrast with the previous study *(50)*, here lower miR-10b expression levels were detected in patients with distant relapse (n = 61), regional relapse (n = 11), and local recurrence (n = 33); these observations suggest that miR-10b does not, in fact, correlate with distant metastasis or poor prognosis *(56)*. Added to these findings, miR-10b expression has been associated with the prognostically favorable luminal A subtype *(25)*. To further elaborate on the findings of these conflicting data, sufficient numbers of patients with long-term follow-up are required to truly elucidate the clinical and functional relevance of miR-10b in breast cancer.

miR-373 and miR-520c. miR-373 and miR-520c have also been associated with breast cancer metastasis. Both in vivo and in vitro studies of MCF-7 cells transduced with an miRNA-expression library showed that subsequent migration and invasion was associated with expression of both miR-373 and miR-520c *(57)*. The ability of cell lines, including those of breast origin (i.e., MDA-MB-435; although whether this cell line is breast or melanoma in origin is still the subject of ongoing debate), to migrate efficiently was also found to depend on endogenous levels of miR-373. In addition, an inverse relationship was identified between the expression of certain miRNAs and CD44 molecule (Indian blood group) (*CD44*), with the suppression of *CD44* significantly correlating with the migratory phenotype of cells that overexpress miR-373 and miR-520c.

miR-21. This miRNA has not only been associated with tumorigenesis *(29–34)*; several investigators have also reported and characterized associations between miR-21 expression and invasive and metastatic breast cancer *(58–60)*. Expression profiling of 435 miRNAs revealed that 9 miRNAs were upregulated (miR-21, miR-29b, miR-29c, miR-98, miR-181b, miR-181d, miR-155, let-7f, miR-365) and 7 were downregulated (miR-30a-3p, miR-31, miR-127, miR-140, miR-320, miR-355, miR-497) in primary breast cancer tissue vs normal adjacent tissue (≥2-fold) *(58)*. miR-21 (validated in 113 primary breast cancer tumors by qPCR) was found to be the most significantly upregulated, correlating with advanced tumor stage, spread to lymph node, and shortened survival time *(58)*. The observed decrease in invasion and metastasis of MDA-MB-231 cells in response to miR-21 inhibition *(59)* and the upregulation of miR-21 levels associated with increased invasion after HER2 overexpression in MDA-MB-435 *(60)* together suggest a relationship between miR-21 expression, HER expression, and cell invasion.

mir-205 and mir-200 family. Reduced/depleted expression of miRNAs with tumor-suppressive roles (see above) has been associated with tumor development and growth. A focus of recent research in this field has been investigation of whether some of these miRNAs may have direct functional impact on metastasis. For example, members of the miR-200 family (i.e., miR-200a, miR-200b, miR-200c, miR-141, and miR-429), along with miR-205, have been reported as downregulated in cells undergoing epithelial-to-mesenchymal transition (EMT), a process considered to be a fundamental step in tumor metastasis *(61)*. The constitutive expression of either the miR-200b-200a-429 cluster or the miR-200c-141 cluster in Madin-Darby canine kidney epithelial cells, via lentiviral transduction, in turn, inhibited transforming growth factor β (TGF-β)-induced EMT *(61)*. In keeping with this observation, downregulation of miR-200b and miR-200c has been demonstrated to be associated with loss of E-cadherin expression in breast cancer cells (MDA-MB-231 and BT-549) with mesenchymal phenotype *(62)*, as a result of a consequential upregulation of the E-cadherin transcriptional repressor, ZEB1. Conversely, miR-200b or miR-200c restoration induced E-cadherin expression, therefore inhibiting EMT and reinstating a less aggressive phenotype in the cancer cells *(62)*.

miR-146a/b. Overexpression of miR-146a and miR-146b via lentiviral infection of MDA-MB-231 cells results in downregulation of nuclear factor-κB (NF-κB), a transcription factor associated with enhanced survival and metastasis of cancer cells *(63)*. Considering

that breast cancer metastasis suppressor 1 (*BRMS1*) is also associated with decreased signaling via the NF-κB pathway and that this metastasis suppressor can induce changes in the expression of miR-146, this miRNA was subsequently proposed to function downstream of BRMS1 *(64)*.

miR-126 and miR-335. miRNA profiling (453 miRNAs) of the MDA-MB-231 parental cell line and its derivatives that are highly metastatic to the bone (BoM1 sublines) or lungs (LM2 sublines) resulted in a focus on 6 miRNAs (miR-122a, miR-126, miR-199a*, miR-206, miR-335, miR-489), whose expression showed the greatest decrease in the metastatic cell lines compared to parental cells. Restoration of miR-335, miR-126, and miR-206 expression in LM2 cells decreased their lung colonization by >5-fold and also decreased bone metastasis *(65)*. miR-126 expression has also been implicated in preventing cell proliferation and tumorigenesis. Unlike miR-126, restoration of miR-335 and miR-206 did not change the rate of proliferation, but was associated with a significant reduction in migratory and invasive capacity in vitro. Several metastasis-associated genes were identified in this study as targets of miR-335, including progenitor cell transcription factor SRY (sex determining region Y)-box 4 (*SOX4*) and extracellular matrix component tenascin C (*TNC*). To determine any clinical association between miR-335, miR-126, miR-206 and metastasis, their expression was evaluated in archived primary breast tumors (n = 20), 11 of which were from patients who suffered bone, lung, or brain (n = 11) metastasis and 9 of which were from patients who did not experience metastatic relapse. qPCR analysis indicated that low-level expression of these miRNAs was associated with a shorter time to relapse. This was found to be especially true for miR-335 and miR-126, whose significantly low expression in tumors was associated with poor overall metastasis-free survival, which suggests that these miRNAs could be metastasis suppressors.

miR-224. A recent study demonstrated that Ubc9 promotes breast cancer cell invasion in MDA-MB-231 cells *(66)*. Specifically, profiling 474 miRNAs in MDA-MB-231 cells overexpressing Ubc9 (an E2-conjugating enzyme that promotes cell invasion and metastasis), showed miR-224 to be significantly downregulated, suggesting a regulatory role for miR-224 on Ubc9. An inverse correlation between Ubc9 and miR-224 expression was supported by Ubc9-siRNA studies, in which an upregulation of miR-224 expression was induced. Furthermore, direct targets of miR-224, namely cell division cycle 42 (GTP binding protein, 25kDa) (*CDC42*) and chemokine (C-X-C motif) receptor 4 (*CXCR4*), were identified, and the suppression of these targets caused an inhibition of Ubc9-mediated invasion. These observations were subsequently confirmed by using 2 other metastatic breast cancer cell lines, MDA-MB-468 and LM2-4142. Thus, these findings suggest that the promotion of metastasis and invasion by Ubc9 is, at least to some extent, directly associated with its downregulation of miR-224 *(66)*.

miR-31. miR-31 is reported as having pleiotropic effects on breast cancer metastasis, and the ability of miR-31 to inhibit multiple steps in the invasion-metastasis cascade has been demonstrated *(67)*. In a study that included MDA-MB-231 and SUM-159 cells, ectopic expression of miR-31 in vitro and in vivo interfered with local invasion, early postintravasation events, and metastatic colonization. Furthermore, miR-31 expression, analyzed in specimens (n = 56) from breast cancer patients, was shown to inversely correlate with subsequent metastasis *(67)*.

Although evidence is mounting that miRNAs have direct relevance in metastasis, such information has only begun to surface, leaving many areas in this exciting field yet to be explored. However, the range of studies reported to date emphasizes the important role that these short RNA transcripts may play in either promoting or preventing cancer progression, a more comprehensive understanding of which is crucial if miRNAs are to be considered to have a potential therapeutic role in breast cancer.

miRNAs Associated with Resistance to Breast Cancer Treatment

POTENTIAL RELEVANCE IN RELATION TO HORMONE TREATMENT

Progression of breast cancer and resistance to endocrine therapies has been attributed to the possibility of hormone-responsive miRNAs involved in the regulation of certain signaling pathways. Focusing on miR-128a, Masri et al. *(68)* reported its selective upregulation in letrozole-resistant MCF-7 cell-line derivatives [testosterone-only MCF-7 cells that are resistant to the aromatase inhibitor letrozole (T+LET R)] compared with MCF-7 cells overexpressing the aromatase gene [cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1*)] (MCF-7aro), with reduced sensitivity of T + LET R cells to TGF-β. To further investigate the possible inverse relationship of miR-128a with TGF-β signaling, the relevance of inhibiting endogenous miR-128a in the T + LET R cells was also investigated, whereby resensitization of these cells to the growth inhibitory effects of TGF-β was observed. The mechanism of TGFβ1 sensitivity loss in T + LET R cells was suggested to result from miR-128a targeting TGFβR1 protein expression. This study concluded that breast

cancer progression and resistance to therapy, owing to the modulation of vital signaling pathways, may be due not only to estrogen-mediated gene expression, but also to hormone-regulated miRNAs *(68)*.

POTENTIAL RELEVANCE IN RELATION TO CHEMOTHERAPY TREATMENT

Studies in MCF-7 suggest that miR-21 may be involved in resistance/reduced sensitivity to topotecan (see Fig. 1), because suppression of miR-21–sensitized MCF-7 cells to its effects. Furthermore, combining taxol treatment with miR-21 inhibition enhances the chemotherapeutic effects of taxol in breast carcinoma cells, suggesting a role for miR-21 in taxane resistance *(69)*. Furthermore, as detailed later, restoration of miR-205 levels in SKBR3 cells apparently eradicates HER3-mediated resistance, improving response to tyrosine kinase inhibitors, Gefitinib and Lapatinib *(46)*.

Although reports on miRNAs associated with response to anticancer treatment have been limited to date, the emerging data give hope that panels of miRNAs will be identified that will contribute to predicting treatment response in breast cancer. Furthermore, these studies suggest a potentially useful combined therapeutic approach for the treatment of breast cancer (see Potential Use of miRNAs as Therapeutic Agents or as Therapeutic Targets for Treatment of Breast Cancer), although this has yet to be fully elucidated.

Potential Use of miRNAs as Biomarkers for Breast Cancer

EXTRACELLULAR CIRCULATING miRNAs

The pursuit of minimally invasive biomarkers is a challenging but exciting area of research. Clearly, such markers would need to be sensitive and specific enough to aid in the detection of breast cancer at an early stage, would monitor progression of the disease, and could predict the individual patient's response to treatment. Existing diagnostic tools for breast cancer, although important and necessary, have many limitations. For example, although mammography is currently considered the more reliable option for earliest diagnosis within certain age groups, associated problems have arisen due to varied interpretation of results among radiologists and inconsistent rates of false-positive results *(70)*. For breast cancer, ER, PR, and HER2 expression are among a very limited number of biomarkers that have been established as relevant for routine assessment *(71)*. Although these receptors have many merits as biomarkers, they arguably cannot be considered as adequate and ideal candidate biomarkers for all breast cancer patients. For example, some patients with HER2-positive tumors do not respond to Trastuzumab, and conversely, some described as having HER2-negative tumors respond to this HER2-targeted monoclonal antibody *(72)*.

Several circulating tumor protein biomarkers frequently used clinically, such as carcinoembryonic antigen and carbohydrate antigen 15-3, have been noted to have low preoperative diagnostic sensitivity, thus indicating their limited use for detecting early-stage breast cancer *(73)*. Over the past decade, certain mRNAs have been reported as circulating in serum/plasma from cancer patients *(74, 75)*. In fact, the feasibility of using whole-genome microarrays and subsequent qPCR as a means of identifying panels of extracellular mRNAs with potential as diagnostic and prognostic breast tumor biomarkers was initially demonstrated in our laboratory *(74)*. Reports on the existence of circulating miRNAs associated with cancer have, however, emerged only within the past 2 years *(76–78)*. The stability of miRNAs, demonstrated to be well preserved in formalin-fixed paraffin-embedded tissue *(79)* prompted researchers to investigate whether such miRNAs exist in blood and other bodily fluids. In 2008, in the first report of miRNAs in serum, increased levels of tumor-associated miRNAs were identified in patients with diffuse large–B-cell lymphoma *(80)*. In our pilot global studies, in addition to circulating mRNAs *(74)*, we observed that circulating miRNAs (including miR-141 and miR-195) were present at significantly higher levels ($P < 0.001$) in serum from patients recently diagnosed with breast cancer compared with age- and sex-matched healthy volunteers (unpublished data). Since then, several very interesting studies have revealed associations between circulating miRNAs and the presence of cancers, including pancreatic *(81)*, colorectal *(82, 83)*, and breast cancer *(84, 85)*.

The concept of miRNAs existing in the systemic circulation extends beyond that of free miRNAs in plasma and serum, because miRNAs (as well as mRNAs and proteins) have been shown to exist in circulating microvesicles *(86, 87)*. For example, exosomes, which are nanosized microvesicles, contain miRNAs and have been associated with cancers, including those of the lung *(88)* and ovary *(89)* as well as glioblastoma *(90)*. Research of circulating miRNAs is still in its infancy; however, evidence from the studies outlined above indicates a positive correlation between these potentially noninvasive biomarkers and cancer diagnosis. Whether these miRNAs exist freely in the systemic circulation or are predominantly transported via tumor-secreted microvesicles remains somewhat unclear, given that evidence supporting both possibilities has been documented. Whichever is the case, further investigation is certainly warranted to increase our understanding of circulating miRNAs as potential diagnostic, prognostic, and predictive biomarkers. If sufficiently confirmed, application of miRNAs to such uses could

provide new hope for the improved management not only of breast cancer, but also of a range of other cancer types.

Potential Use of miRNAs as Therapeutic Agents or as Therapeutic Targets for Treatment of Breast Cancer

The growing list of reports indicating the significance of miRNAs in diagnosis and prognosis of breast cancer has led to the advancement of research to explore the potential relevance of miRNAs as therapeutics (see Fig. 1 for a summary of data available on miR-21 as an example). An attractive attribute of miRNAs is their ability to target gene networks at multiple levels *(91)*. This finding, together with the observations that miRNAs can either promote or suppress tumorigenesis and metastasis, and have been found to be associated with tumor subtype and with response to systemic therapy, has stimulated efforts to modulate miRNA expression to reduce tumor development and metastasis, improve response to treatment, and prevent resistance to therapy.

Several methods exist that may be used to adjust miRNA expression, depending on whether the objective is to inhibit the expression of oncogenic miRNA(s) or to increase the expression of tumor-suppressive miRNA(s). Antisense oligonucleotides or their chemically tailored analogs (known as locked nucleic acids) may be used to inactivate an oncogenic miRNA; targeting the precursors of mature miRNAs also can inhibit production *(92)*. Such modalities may be considered as part of a treatment regime that also includes other small molecule/monoclonal antibody-targeted agents or chemotherapy. Alternatively, to increase the expression of a tumor suppressing miRNA (as a miRNA replacement-therapy), viral or liposomal delivery methods are showing promise in achieving miRNA introduction [as reviewed in *(93)*]. However, alternate approaches such as repetitive administration of miRNAs and exploitation of antibody-mediated endocytosis are also being considered as optimal routes for the delivery of small RNA therapeutics.

Although the therapeutic delivery of any new molecular type involves challenges in getting acceptance to move from preclinical to clinical investigation, miRNAs have the advantage that much of the fundamental work in this field is already at an advanced stage with regard to the delivery of antisense and siRNA therapeutics. Major clinical advances are currently underway in the ribonucleic-acid–based arena. For example, a phase II trial is currently underway with an siRNA molecule termed ALN-RSV01 (owned by Alnylam) for the treatment of respiratory syncytial virus infections. Bevasiranib (an Opko Health agent) has recently entered a phase III trial for the treatment of wet age-related macular degeneration, after phase II trials proved it to be safe and well tolerated, and to afford substantial benefits with respect to near vision and lesion size. A number of other RNA molecules are currently in phase I clinical trials, e.g., AKli-5 (Quark Biotech) for the treatment of acute renal failure. Others are in phase II clinical trials, e.g., RTP801i-14 (Silence Therapeutics) for wet age-related macular degeneration.

Of course, development of the optimal delivery solutions for miRNA therapeutics as anticancer agents has its own challenges (e.g., identifying cancer cell–specific antigens against which to target antibodies for steering delivery), and this remains an important component in realizing such a therapeutic approach. miRNAs have the advantage that siRNA delivery has at least paved the way for the acceptance of therapeutic use of small RNA molecules. Undoubtedly the relevance and potential of RNA-based therapeutics has been realized by pharmaceutical and biotechnology companies. For example, Isis has licensed its preclinical antisense programs in diabetes, obesity, and metabolic disease to Bristol-Myers Squibb (at a cost of $192 million) and to Ortho-McNeil/Johnson and Johnson (for $460 million). Archemix has partnered with Elan (in a $360 million deal), as well as with Pfizer, Takeda, and Merck Serono on its aptamer program. Merck has acquired Sirna for $1.1 billion. These developments instill a level of confidence, indicating that although much has yet to be done to routinely translate small RNAs such as miRNAs to the clinic, there is a commitment to do so.

Several of the studies discussed in this review have indicated the potential for altering expression of specific miRNAs as a means to identify their function and/or their potential therapeutic relevance. The inhibition of miR-21 expression in MCF-7 breast cancer cells is a prime example of how antisense oligonucleotides may be used to manipulate the expression of an oncogenic miRNA *(29)*. miR-21 expression, known to be increased in breast cancer, has also been implicated in the modulation of the tumor suppressor, PTEN (phosphatase and tensin) *(33)*, as well as a number of other targets (as previously mentioned: see miR-21). miR-21 therefore may be an attractive therapeutic target, because PTEN has been reported to be a regulator of sensitivity to Trastuzumab *(94)* (See Fig. 1). This concept is further supported (as summarized in Potential Relevance in Relation to Chemotherapy Treatment) because suppression of miR-21 has been shown to sensitize breast cancer cells to topotecan and taxol. However, miR-21 is not the only miRNA whose manipulation may be of benefit in sensitizing breast cancer cells to anticancer therapy. For example, miR-205 introduction into such cells increases response to

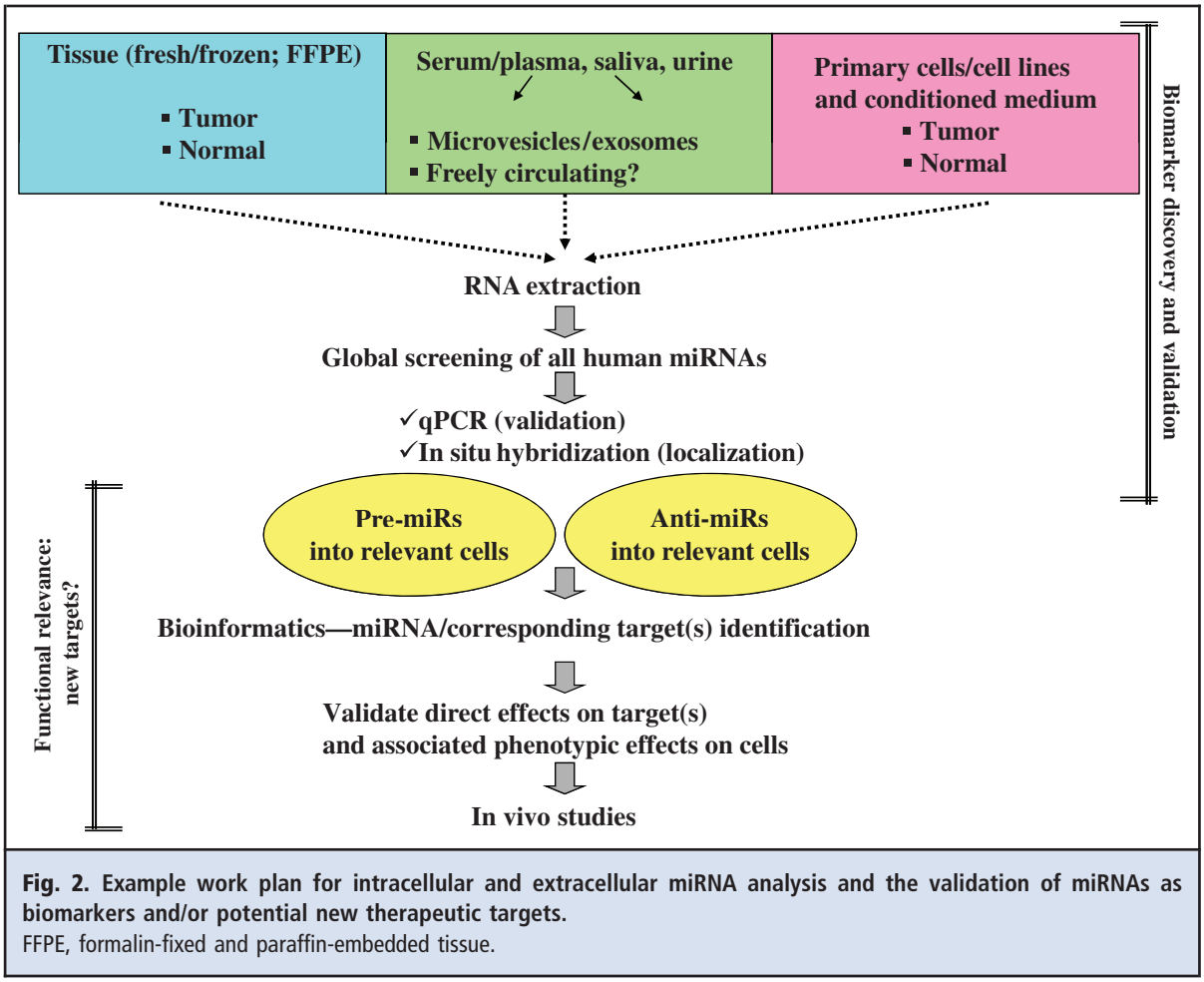

Fig. 2. Example work plan for intracellular and extracellular miRNA analysis and the validation of miRNAs as biomarkers and/or potential new therapeutic targets.
FFPE, formalin-fixed and paraffin-embedded tissue.

Gefibinib and Lapatinib. Collectively, these observations support the possibility that modulating (singly or multiply) miRNA expression—through administration of, for example, premirs or antimirs—may be of clinical benefit when combined with chemotherapy or more targeted therapies.

These are but a few examples of how the exploitation of miRNA expression may be applicable to the future treatment of breast cancer. Although research in this area looks promising, the transition from bench to bedside still faces several obstacles. The functional validation of breast cancer–associated miRNAs is still at an early stage. The identification of further clinically relevant miRNAs will undoubtedly be important. To fully comprehend the significance of these short RNAs, it is imperative that all human miRNAs, in addition to those currently known, are identified and characterized in full. Standardization of techniques involved in miRNA analysis and comprehensive collaborative studies (such as those we propose in Fig. 2), with due consideration given to technical issues (e.g., type and number of miRNAs to be examined, experimental conditions, test sensitivity, specificity) are necessary if miRNAs are to reach their full potential in the clinical setting *(9)*.

In conclusion, since their initial discovery of miRNAs in 1993, the first discovery of their association with cancer in 2002, and the subsequent identification of their presence in the systemic circulation in 2008, miRNAs have revolutionized our understanding of cancer biology. The tumor suppressive and oncogenic roles of these molecules in certain cancers has stimulated numerous investigations regarding how such miRNAs may be used as biomarkers and possibly manipulated for clinical benefit. Our knowledge in this field of research is increasing rapidly. The prospect that circulating miRNAs (whether freely existing or in microvesicles) may be useful as diagnostic, prognostic, and/or predictive biomarkers—some of which may also have relevance as new therapeutic targets—looks promising and very exciting. Yet to be fully explored, however, is the potential benefit of miRNAs in these

settings, taking into consideration limitations and realistic use. The power and potential to truly exploit miRNAs and translate this information to the clinic in the interest of breast cancer patients will depend on further investigations that fully characterize such miRNAs, their functional targets, and the phenotypic effects associated with their targeted manipulation. Moving forward, for reliable outcomes to be reached the associated collaborative translational studies should involve the inclusion of large cohorts of consenting breast cancer patients (representing all subtypes) and healthy volunteers. For example, such studies are possible in Ireland as facilitated by ICORG (All Ireland Cooperative Oncology Research Group) as well as through international collaborations. In the relatively short time since their discovery, miRNAs have shown great potential, both as tumor biomarkers and potentially as therapeutic targets, to be important contributors to the future management of cancers, including breast cancers. Such considerations provide an excellent basis on which to build future studies.

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