

MicroRNAs as Prognostic Indicators and Therapeutic Targets: Potential Effect on Breast Cancer Management

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Abstract The discovery of microRNAs (miRNA) as novel modulators of gene expression has resulted in a rapidly expanding repertoire of molecules in this family, as reflected in the concomitant expansion of scientific literature. MiRNAs are a category of naturally occurring RNA molecules that play important regulatory roles in plants and animals by targeting mRNAs for cleavage or translational repression. Characteristically, miRNAs are noncoding, single-stranded short (18-22 nucleotides) RNAs, features which possibly explain why they had not been intensively investigated until recently. Accumulating experimental evidence indicates that miRNAs play a pivotal role in many cellular functions via the regulation of gene expression. Furthermore, their dysregulation and/or mutation has been shown in carcinogenesis. We provide a brief review of miRNA biogenesis and discuss the technical challenges of modifying experimental techniques to facilitate the identification and characterization of these small RNAs. MiRNA function and their involvement in malignancy, particularly their putative role as oncogenes or tumor suppressors is also discussed, with a specific emphasis on breast cancer. Finally, we comment on the potential role of miRNAs in breast cancer management, particularly in improving current prognostic tools and achieving the goal of individualized cancer treatment.

The molecular etiology of malignancy remains one of the most challenging disease processes under scientific investigation. The incidence of malignancy such as breast cancer is increasing worldwide, with cancer replacing heart disease as the leading cause of disease-related mortality (1). Initially identified in 1993, the first microRNAs (miRNA; *lin-4* and *let-7*) were linked to temporal control of developmental events in *Caenorhabditis elegans* (2). MiRNAs are now recognized as a highly abundant class of regulatory RNA molecules. The importance of miRNA-directed gene regulation is accentuated as more miRNAs and their regulatory targets and functions are discovered. At the time of writing, 3,834 mature miRNA sequences have been described in primates, rodents, birds, fish, worms, flies, plants, and viruses (3). In the human genome, 474 mature miRNAs have been reported; however, computational prediction estimates that this could increase to >1,000 (4).

miRNA biogenesis is a multistage process commencing in the nucleus when primary (pri-) miRNAs are transcribed by RNA polymerase II (Fig. 1; refs. 5, 6). Pri-miRNA transcription occurs from distinct genomic locations; many are intergenic with independent promoters, others are clustered in polycistronic transcripts, redolent of coordinate regulation. Certain pri-miRNAs are located within introns of host genes suggesting that transcriptional regulation of miRNA biogenesis may be under host-gene promoter control in some instances (6, 7). Pri-miRNAs

are processed to shorter ~70 to 85 nucleotide precursor (pre-) miRNAs, consisting of an imperfect stem-loop structure. Precursor molecules are subsequently exported to the cytoplasm and cleaved to produce a transient miRNA duplex composed of a mature miRNA sequence (~22 nucleotides in length) and its' complementary sequence miRNA*. This sequential miRNA processing is mediated by RNase III endonucleases Drossha and Dicer and their protein binding partners, DGCR8 and TRBP, respectively (Fig. 1). A miRNP (miRNA-containing ribonucleoprotein particle) complex containing Argonaute protein (Ago2) separates the miRNA duplex by binding the strand with the less stable hydrogen bonding at its 5'-end. This strand becomes the mature, functionally active miRNA (8). The other strand is degraded.

MiRNAs exhibit high evolutionary conservation over a wide range of species and exhibit diversity in expression profiles in cell types suggesting important regulatory functions (9). MiRNAs exert their functionality via sequence-specific regulation of posttranscriptional gene expression by targeting mRNAs for cleavage or translational repression (10). Most animal miRNAs bind to the 3'-untranslated region (UTR) of target mRNA transcripts, in which cis-regulatory sequences of many known posttranscriptional events occur. However, the binding of miRNAs to coding or 5'-UTRs can also be functional (11), although this has not yet been clearly established in mammalian systems. The miRNP complex, similar to the RNA-induced silencing complex, is guided to the mRNA target site by the miRNA strand. Perfect or near-perfect complementarity to the target site can induce gene silencing via the RNA interference pathway (12); causing cleavage of the mRNA transcript and degradation of the target mRNA. Predominantly, however, the miRNA-mRNA interaction is imperfect, resulting in translational repression and subsequent reduction of steady state protein levels of targeted genes (6).

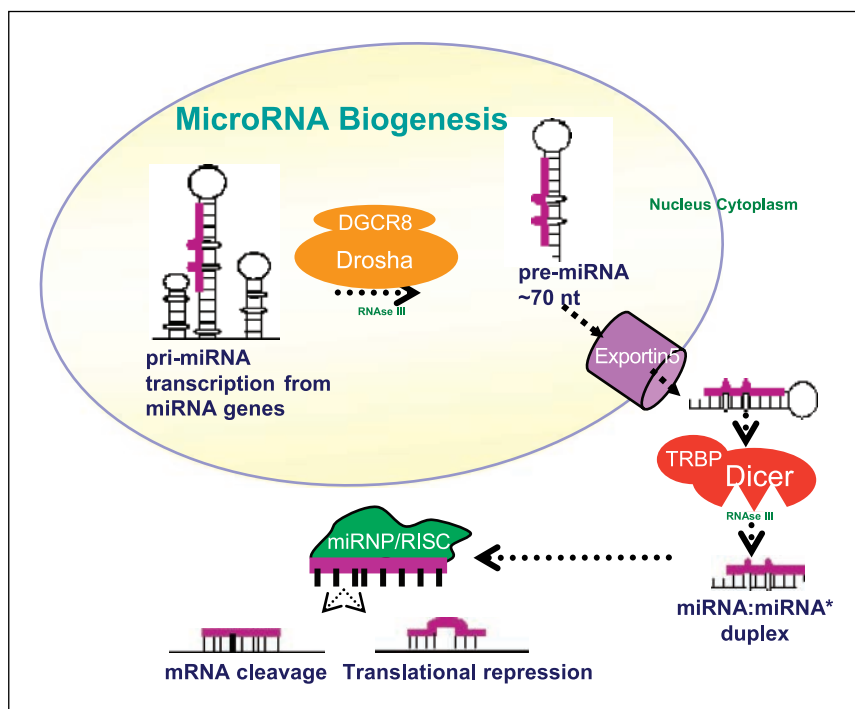
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Fig. 1. miRNA biogenesis and processing. This is a simplified representation of miRNA biogenesis and processing in human cells. The multistep process begins in the nucleus where the RNase III enzyme Drosha, coupled with its binding partner DGCR8, cleaves nascent miRNA transcripts (pri-miRNA) into ~70 nucleotide precursors (pre-miRNA). These pre-miRNAs consist of an imperfect stem-loop structure. Pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5. In the cytoplasm, the hairpin precursors are cleaved by Dicer and its binding partner the transactivator RNA-binding protein TRBP into a small, imperfect dsRNA duplex (miRNA:miRNA*) that contains both the mature miRNA strand and its complementary strand. The miRNA strand is incorporated into the miRNP complex and targets complementary mRNA sequences, exerting its functionality via mRNA cleavage or translational repression.



The precise mechanism of translational repression has not been definitively elucidated (10) and may occur before or after the initiation of translation (13, 14). Associated reduction in mRNA abundance has also been reported, however, it is unclear whether this is related to translational repression or is a separate miRNA regulation mechanism; translationally silenced mRNAs may be sequestered in cytoplasmic P-bodies containing mRNA degradation enzymes (15). Alternatively, de-adenylation and decapping of targeted mRNAs can occur independently of translational repression (16).

There is considerable evidence that small interfering RNAs, play a role in transcriptional gene silencing via epigenetic changes including DNA methylation and histone modification (17). Small interfering RNAs and miRNAs share similar processing pathways and exert posttranscriptional effects via RNA interference. The similarities between miRNA and small interfering RNA suggest that miRNAs also have the potential to affect epigenetic mechanisms including methylation and histone deacetylation (18).

The imperfect nature of the interaction between a miRNA and its target makes the prediction of candidate miRNAs particularly challenging. The specific region of miRNA important for mRNA target recognition is located in the 5'-end of the mature miRNA from bases 2 to 8, often referred to as the "seed-sequence" (6). Computational target prediction algorithms have been developed to identify putative miRNA targets and place considerable importance on this seed-sequence, using it to search for complementary sequences in the 3'-UTRs of known genes that exhibit conservation across species. Such algorithms have predicted that each miRNA may potentially bind to as many as 200 targets, and it is estimated that miRNAs control the expression of *at least* one third of human mRNA (19). These methods are limited, however, by the fact that not all miRNAs target the 3'-UTR (11), and some biological processes mediated by the miRNA-target interaction may be

specific to humans (20); the use of conservation as a limiting criterion may result in biologically significant targets being missed. For this reason, additional methods of target prediction should be considered, including the use of experimental techniques such as observation of mRNA down-regulation following the overexpression of specific miRNAs (21). Experimental validation and functional analysis is essential before a mRNA can truly be regarded as a miRNA target.

MiRNA gene regulation plays key roles during development and in various cellular processes such as differentiation, cell growth, and cell death (6). These processes are commonly dysregulated in cancer, implicating miRNAs in carcinogenesis. The first discovery of a link between miRNAs and malignancy was the identification of a translocation-induced deletion at chromosome 13q14.3 in B-cell chronic lymphocytic leukemia (22). Loss of miR-15a and miR-16-1 from this locus results in increased expression of the antiapoptotic gene *BCL2*. Accelerating research in this field has resulted in the identification and confirmation of abnormal miRNA expression in a number of human malignancies including breast cancer (Table 1).

Experimental Techniques for miRNA Analysis

The unique size and structure of miRNAs necessitated the modification of existing techniques to facilitate their analysis. Traditional column-based RNA extraction techniques, adopted to selectively isolate high-quality large RNA, led to miRNA being discarded. Adaptation of existing protocols was required to capture the miRNA component.

Microarray technology has also been modified to facilitate miRNA expression profiling. Castoldi et al. (23) described a novel miRNA microarray platform using locked nucleic acid-modified capture probes. Locked nucleic acid modification improved probe thermostability and increased specificity, enabling miRNAs with single nucleotide differences to be

discriminated—an important consideration as sequence-related family members may be involved in different physiologic functions (24). An alternative high-throughput miRNA profiling technique is the bead-based flow cytometric approach developed by Lu et al. (25); individual polystyrene beads coupled to miRNA complementary probes are marked with fluorescent tags. After hybridization with size-fractionated RNAs and streptavidin-phycoerythrin staining, the beads are analyzed using a flow-cytometer to measure bead color and phycoerythrin, denoting miRNA identity and abundance, respectively. This method offers high specificity for closely related miRNAs because hybridization occurs in solution. The high-throughput capability of array-based platforms make them an attractive option for miRNA studies compared with lower throughput techniques such as Northern blotting and cloning, which remain essential for the validation of microarray data.

Quantitative real-time PCR methodologies have been widely applied to miRNA research. To date, the most successful approach in terms of specificity and sensitivity is a two-step approach using looped miRNA-specific reverse transcription primers and TaqMan probes from Applied Biosystems. Multiplex PCR approaches for miRNAs have also been reported (26).

The development of methods to manipulate miRNA expression has enabled investigation into the cellular processes affected by differentially expressed miRNAs and their mRNA targets. 2-O-Methyl antisense single-strand oligonucleotides (27–29) and locked nucleic acid-modified oligonucleotides (30) have been developed as miRNA inhibitors, making the suppression of endogenous miRNA activity and its downstream effect on mRNA expression achievable both *in vitro* and *in vivo*. The effect of target miRNA knockdown on cell morphology and function can be determined using standard assays for processes such as cell proliferation, migration, invasion, and angiogenesis. The effects of miRNA inhibition can be studied in animal models via transfection with tumor cells treated with miRNA inhibitors (31) or by the intravenous injection of “antagomirs” (2-O-methyl-modified nucleotides with a cholesterol moiety at the 3'-end; ref. 32).

MiRNA mimicry, a complementary technique to inhibition, has recently been used *in vitro* to identify cellular processes and

phenotypic changes associated with specific miRNAs transfected into cell lines. Functional assays (e.g., proliferation, migration, invasion, and angiogenesis) can be done to determine the effect of miRNA up-regulation on tumorigenic or nontumorigenic cell populations.

miRNA and Breast Cancer

Despite the dedication of research and resources to the elucidation of the molecular mechanisms involved, unpredictable response and development of resistance to adjuvant therapy remain major challenges in the management of breast cancer patients. The emergence of miRNAs as regulators of gene expression identifies them as obvious novel candidate diagnostic and prognostic indicators and therapeutic targets.

Calin et al. (33) showed that half of the known miRNAs are located in cancer-associated genomic regions/fragile sites, thus potentiating their role in cancer. A specific example of this is the polycistron cluster miR-17-92 at the *c13orf25* locus on chromosome 13q31. This locus is known to undergo loss of heterozygosity in a number of different cancer types, including breast cancer (34). Similarly, miR-125b, which is underexpressed in breast cancer, is located at chromosome 11q23-24, one of the regions most frequently deleted in breast, ovarian, and lung tumors (34). A number of other miRNAs, e.g., miR-196 and miR-10a are located in Homeobox clusters (33), known to be involved in the development of breast cancer and malignant capacity of cancer cells (35).

MiRNA expression profiles have been used to differentiate tumor tissue from surrounding normal tissue for tumor classification and for prognostication. Iorio et al. (36) did a microarray analysis of 76 breast tumor and 34 normal specimens; they identified 29 miRNAs as deregulated in breast cancer, and a set of 15 miRNAs that could correctly predict the nature of the sample analyzed. In addition, miRNA expression was correlated with biopathologic features such as estrogen receptor (ER) and progesterone receptor status (miR-30) and tumor stage (miR-213 and miR 203). The differential expression of several let-7 isoforms was associated with biopathologic features including progesterone receptor status (let-7c), lymph

Table 1. Some miRNAs with altered expression in malignancy

| Tissue/tumor type | Increased expression | Decreased expression |
|------------------------------|--|--|
| Lung | mir-17-92cluster, miR-17-5p | let-7 family |
| Breast | miR-21, miR-29b-2 | miR-125b, miR-145 miR-10b, miR-155, miR-17-5p, miR-27b |
| Glioblastoma | miR-221, miR-21 | miR-181a, miR-181b, miR-181c |
| Chronic lymphocytic leukemia | | miR-15, miR-16 |
| Lymphoma | miR-155, miR-17-92cluster | miR-15a |
| Colorectal | miR-10a, miR-17-92 cluster, miR-20a, miR-24-1, miR-29b-2, miR-31 | miR-143, miR-145, let-7 |
| Thyroid | miR-221, miR-222, miR-146, miR-181b, miR-197, miR-346 | |
| Hepatocellular | miR-18, miR-224 | miR-199a, miR-195, miR-200a, miR-125a |
| Testicular | miR-372, miR-373 | |
| Pancreatic | miR-221, miR-376a, miR301, miR-21, miR-24-2, miR-100, miR-103-1,2, miR-107, miR-125b-1 | miR-375 |
| Cholangiocarcinoma | miR-21, miR-141, miR-200b | |
| Prostate | let-7d, miR-195, miR-203 | miR-128a |
| Gastric | miR-223, miR-21, miR-103-2 | miR-218-2 |

node metastasis (let-7f-1, let-7a-3, and let-7a-2), or high proliferation index (let-7c and let-7d) in breast tumor samples (36). Mattie et al. (37) identified unique sets of miRNAs associated with breast cancers currently defined by their Her2neu/ErBB2 status or their ER/progesterone receptor status. Significantly, there was overlap between the miRNAs identified in these profiles and Iorio's panel (36). In initial studies, we have shown that the expression levels of miR-195 and mir-154 are negatively correlated with ER positivity in a cohort of early breast cancers (38).

The capacity of miRNA expression profiles to classify breast tumors according to clinicopathologic variables currently used to predict disease progression highlights the potential of miRNA signatures as novel prognostic indicators which may contribute to the improved selection of patients for adjuvant therapy. This is an approach that has already shown promise with genomic signatures (39), and miRNA profiles may have superior accuracy to mRNA profiling in this regard (25). Clustering of breast cancer tumors according to Her2neu/ErBB2 status or ER/progesterone receptor status, as shown by Mattie et al. (37), is of particular relevance; mRNA profiles have identified distinct molecular subclasses of breast cancer, predictive of prognosis, based on their ER and Her2neu/ErBB2 classification (luminal A, luminal B, basal-like, Her2-overexpressing, normal-like; ref. 40). A comprehensive interrogation of the breast cancer subclasses via miRNA expression profiling could further characterize the molecular basis underlying these subtypes, perhaps define more precise subsets of breast cancer, and provide opportunities for the identification of novel targets that can be exploited for targeted therapy.

Specific Roles of miRNAs in Breast Cancer

MiRNAs can function as oncogenes via negative inhibition of tumor suppressor genes or interference in pathways that control cellular differentiation or apoptosis. Si et al. (31) identified miR-21 as an oncogenic miRNA and showed that introduction of anti-miR-21 significantly reduced miR-21 expression in MCF-7 breast cancer cells. This resulted in reduced cell growth, in a dose-dependent manner, due to decreased proliferation and increased apoptosis. Furthermore, using a xenograft carcinoma mouse model, it was found that MCF-7 cells transfected with anti-miR-21 grew substantially slower than negative controls when injected into mouse mammary pads. In our laboratory, human breast cancer primary tumors showed increased expression of miR-21 compared with normal breast tissues (38).

Conversely, miR-17-5p is expressed at low levels in breast cancer and was identified by Hossain et al. (41) as a probable tumor suppressor miRNA mediated by the down-regulation of the AIB1 protein, which is known to be overexpressed in breast cancer. MiRNAs have also been shown to regulate the hormone and epidermal growth factor receptors currently used as prognostic indicators and therapeutic targets in breast cancer. Adams et al. (42) examined the relationship between miR-206 (shown to be elevated in ER α -negative tumors; ref. 36), and the expression of ER α . The mechanism of ER α silencing in certain tumors remains equivocal. The authors identified and verified two specific miR-206 binding sites in the ER α 3'-UTR. Transfection of MCF-7 breast cancer cells with synthetic pre-miR-206 induced a dose-dependent repression of ER α mRNA

levels. Conversely, MCF-7 cell transfection with antagomiR-206 resulted in increased ER α mRNA, indicating that miR-206 regulates endogenous ER α mRNA levels. Furthermore, treatment with ER α -selective agonists decreased miR-206 levels within MCF-7 cells. Notably, this study detected at least 65 putative miRNA target sites in the 3'-UTR of the ER α transcript, suggesting that multiple miRNAs may play a role in regulation of ER α expression. The existence of a feedback loop between miR-206 and estradiol has considerable implications for our understanding of the endocrine influence on breast cancer, and the mechanisms involved in hormonal therapy resistance.

Scott et al. (43) showed that retroviral overexpression of miR-125a and miR-125b in ERBB2-dependent human breast cancer cell line SKBR3 resulted in the down-regulation of ERBB2 and ERBB3 mRNA and protein levels, suppression of anchorage-dependent growth potential, and inhibition of motility and invasive capabilities. In addition, signaling by the ERK1/2 and AKT pathways was inhibited. Her-2neu/ERBB2-overexpressing breast tumors exhibit aggressive growth and unpredictable response to therapy; enhanced understanding of the regulation of ERBB2 expression has the potential to greatly improve the management of these aggressive tumors.

Therapeutic Potential

The association of miRNA dysregulation with tumorigenesis and the functional analysis of specific miRNAs illustrates the feasibility of using miRNAs as targets of therapeutic intervention.

Anti-miRNA 2-O-methyl or locked nucleic acid oligonucleotides used to inactivate oncomirs such as miR-21 in breast tumors may taper tumor growth (31). Anti-miR-21-induced reduction in tumor growth is potentiated by the addition of the chemotherapeutic agent topotecan, an inhibitor of DNA topoisomerase I, suggesting that suppression of the oncogenic miR-21 could sensitize tumor cells to anticancer therapy, which is an exciting prospect for patients exhibiting a poor response to primary chemotherapy.

Conversely, the induction of tumor suppressor miRNA expression using viral or liposomal delivery of tissue-specific tumor suppressors to affected tissue may result in the prevention of progression or even shrinking of breast tumors.

Tumor suppressor miRNA induction has also been shown to be subject to epigenetic control. Using chromatin remodeling drugs to simultaneously inhibit DNA methylation and histone deacetylation, epigenetic alterations in cancer and normal cells were manipulated by Saito et al. (44), who showed that certain miRNAs were up-regulated in tumor cells but not in normal cells. MiR-127, which exhibited reduced expression in 75% of human cancer cells tested, was significantly up-regulated after treatment. The induction of this miRNA was associated with down-regulation of the proto-oncogene BCL6, suggesting a cancer-protective effect for miR-127 and a novel therapeutic strategy for the prevention and treatment of malignancy.

miRNAs and Stem Cells: Further Therapeutic Potential

Breast tumors contain a heterogeneous population of cells, a minority of which have been designated breast cancer stem cells. These cells are implicated in tumorigenesis due to their

ability to self-renew and proliferate. Deregulation of the self-renewal process leading to stem cell expansion is a likely early event in carcinogenesis (45). The ability of miRNAs to simultaneously regulate many target genes makes them attractive candidates for regulating stem cell self-renewal and cell fate decisions. Accumulating evidence supports this hypothesis; specific miRNAs are differentially expressed in stem cells (46). The ablation of Dicer, a critical enzyme in miRNA generation, is lethal to mouse embryos and results in loss of stem cell populations (47), whereas embryonic stem cells lacking Dicer have been specifically shown to be defective in proliferation and differentiation (48). Furthermore, miRNAs are required to enable stem cells to overcome the G₁-S checkpoint and achieve self-renewal (49). The identification and manipulation of such miRNAs would facilitate the regulation of the stem cell population driving tumorigenesis.

An additional stem cell/miRNA therapeutic possibility is the use of mesenchymal stem cells as potential tumor-targeted vectors for miRNA therapeutics. The ability of mesenchymal stem cells to home directly to breast tumors and nodal metastases has been shown *in vivo* (50). If mesenchymal stem

cells could be used to deliver miRNA antisense strands or antagomirs directly to the tumor site, this may reduce the problem of potential off-target effects of miRNA knockdown elsewhere in the body.

Uncovering the precise role of miRNAs in the regulation of cancer stem cell renewal, and the potential for combination of stem cell and miRNA-based therapeutics provides exciting implications for the future management of breast cancer.

Conclusion

The involvement of miRNAs in the initiation and progression of human malignancy holds much potential for new developments in current diagnostic and therapeutic strategies in the management of patients with breast cancer. The identification of novel miRNAs, the elucidation of their mRNA targets, and an understanding of their functional effects will improve our knowledge of the roles of these novel biomarkers in carcinogenesis and open avenues for potential therapeutic intervention.

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