Involvement of MicroRNAs in Breast Cancer

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ABSTRACT

MicroRNAs regulate numerous aspects of normal and pathologic cellular processes, including cancer. Breast cancer is a heterogeneous form of cancer that is derived from mammary epithelial cells. This review discusses the involvement of microRNAs in the regulation of normal mammary epithelial stem cells, their differentiation into basal and luminal phenotypes, and their control of breast *cancer stem cells*, also referred to as *tumor-initiating cells*. In the second section, we summarize the findings of differential microRNA expression in normal versus breast tumor tissue and among the various subtypes of breast cancer (primarily *luminal*, *basal-like*, and *HER2*). In the third and fourth sections of the review, specific mRNA targets of microRNAs in breast cancer are discussed, including those encoding the estrogen receptor-α and epidermal growth factor receptor, as well as survival, tumor suppressor, and cell-cycle–related proteins. Finally, the involvement of microRNAs in the promotion and suppression of breast cancer metastasis is reviewed. The studies presented herein provide a rationale for the design of therapeutic agents that target specific microRNAs in the treatment of breast cancer. Hopefully, this review will provide an impetus for more studies on the role of microRNAs in the regulation of normal mammary gland development and function.

KEYWORDS: Breast cancer, microRNA, stem cell, hormone receptor, metastasis

Breast cancer, which is the most frequent neoplasm in women worldwide, is a disease that displays heterogeneous phenotypes among individual patients.[1] Accurate assessment of these different phenotypes is crucial to accurate diagnosis, selection of treatment modalities, and prognosis. With the advent of the "omics" approach to the study of disease, there has been an active attempt to understand the global changes in gene expression that underlie given phenotypes of breast cancer. As discussed in this review, mRNA profiling has advanced the diagnosis and treatment of breast cancers through the ability to assign individual tumors to specific subtypes.[1–3]

It is now well established that microRNAs (miRNAs) represent an important determinant of global mRNA expression in normal and diseased tissue,[4] including cancers.[5–7] Because a single miRNA regulates the expression of multiple proteins, miRNAs are likely to emerge as a better diagnostic parameter than messenger RNAs (mRNAs) and as a more effective target of selective therapeutic modalities.

With a very few notable exceptions, little is known concerning the role of miRNAs in normal breast development, function, and involution. Consequently, this review is limited primarily to the functions of miRNA in neoplastic mammary epithelial tissue. Because of space constraints, only primary literature involving breast and miRNAs is referenced, and we apologize to those whose work is not cited directly.

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Emerging Role of MicroRNAs in Reproductive Medicine; Guest Editor, Nasser Chegini, Ph.D.

Semin Reprod Med 2008;26:522–536.
DOI 10.1055/s-0028-1096132. ISSN 1526-8004.

miRNAs AND MAMMARY STEM CELLS

The mammary epithelium is largely a two-layered, stratified epithelium.[8] The luminal layer of cells is composed of either ductal or alveolar cells. The basal layer is composed of myoepithelial cells, which secrete the basal lamina, and the specialized stem cell population. Current models of mammary epithelial populations and their renewal/differentiation propose a hierarchal configuration that begins with mammary stem cells.[8–10] The evidence for such stem cells in both mouse and human are derived from studies in which mammary epithelial cells are selected based on several markers, followed by both expansion in vitro (e.g., mammosphere cultures) and by growth and differentiation into all elements of the mammary epithelial tree after transplantation into cleared mouse fat pads.[11,12]

The possibility that miRNAs are differentially expressed in mammary epithelial stem cells was addressed by Ibarra and colleagues,[13] who studied a subpopulation (Sca-1^{high}) of the pluripotential murine mammary Comma-Dβ cells. Ibarra et al[13] used aldehyde dehydrogenase (ALDH) activity as a marker of stem cells. Selection of an $ALDH^{bright}$Sca-1^{high} subpopulation generated cells that had a high frequency of colony formation, gave rise to both luminal and myoepithelial cells, and efficiently formed mammospheres in vitro. As ALDH confers resistance to certain antitumor drugs, Comma-Dβ cells were treated with mafosfamide (MAF), which produced a 15-fold enrichment of the $ALDH^{bright}$Sca-1^{hi} subpopulation.

Ibarra et al[13] analyzed miRNA profiles in expression libraries constructed from Sca-1^{high}, Sca-1^{neg}, $ALDH^{bright}$Sca-1^{high}, and MAF-treated cells. Two miRNAs, miR-205 and miR-22, were expressed at a high level in the $ALDH^{bright}$Sca-1^{high} progenitors. The authors note that miR-205 has been shown to be highly expressed in breast and thymus and that both miR-205 and miR-22 genes harbor Nanog- and Sox2-binding sites near their promoter regions. More work is needed to determine whether the high levels of miR-205 and miR-22 are functionally related to the maintenance of "stemness" in breast cells. It is worth noting that miR-205 was detected in normal basal epithelial cells by in situ hybridization,[14] indicating that expression of this miRNA does not appear to be limited to stem cells. Interestingly, high levels of miR-205 were associated with a poor prognosis for basal-type breast cancer,[14] suggesting that miR-205 may be enriched in tumor-initiating cells (see next section). Other miRNAs, notably let-7b, let-7c, and miR-93, were expressed at high levels in non-stem, Sca-1^{neg} cells[13] (Table 1). These investigators[13] also demonstrated that activation of the Wnt-1/frizzled/β-catenin signaling pathway increased ALDH expression along with miR-205. Conversely, forced expression of let-7c in Comma dβ cells decreased the $ALDH^{bright}$ population by 6-fold and induced the appearance of Sca-1^{neg} and Sca-1^{lo} compartments—evidence that let-7 is functionally involved in the differentiation of stem cells. Finally, Ibarra et al[13] demonstrated that inserting the perfect complement of let-7c into the 3′ untranslated region (3′ UTR) of the cDNA encoding the red fluorescent protein, DsRed, provided a useful marker for the selection of stem versus differentiated cells. In summary, this study clearly demonstrated changes in miRNA expression in progenitor versus differentiated mammary epithelial cells and opens up a direction of research into the actual roles and mRNA targets of breast stem cell–related miRNAs.

INVOLVEMENT OF miRNAs IN BREAST CANCER STEM CELLS, OR TUMOR-INITIATING CELLS

Cancer stem cells, or tumor-initiating cells (T-ICs), have been tentatively identified in several forms of cancer, including breast cancer.[15] The paradigm of T-ICs is similar to that of normal stem cells described above, in that a subpopulation of "stem-like" cells have the ability to both self-renew, probably at a relatively slow rate, and to give rise to "differentiated" cells (e.g., basal- or luminal-like, estrogen receptor (ER)α-positive or ERα-negative, etc.). Unlike normal stem cells, T-ICs have sustained a sufficient degree of genetic damage to allow them to be robustly tumorigenic and metastatic.[15] Despite many uncertainties concerning T-ICs in breast cancer, the concept of T-ICs is prompting a new outlook on breast cancer development and treatment. Targeting "differentiated" cells will cause the tumor to regress, but if breast T-ICs remain, they may lead to recurrence and/or resistance to therapy. In fact, chemotherapeutic agents may select for breast T-ICs, as discussed above.[13]

Yu et al[16] recently provided evidence for the negative regulation of "stemness" by miRNAs in breast T-ICs. Enrichment for breast T-ICs in the human breast cancer SK-BR-3 cell line was attained through three passages of tumorigenesis in epirubicin-treated immunodeficient mice. These cells, called SK-3^{rd} cells, efficiently generated nonadherent mammospheres in culture, with more cells/mammosphere. Mammospheric SK-3^{rd} cells also efficiently gave rise to tumors and generated micrometastases in the lungs and livers of mice. In addition, the SK-3^{rd} cells gave rise to both basal and luminal mammary epithelial cell types under differentiation-inducing culture conditions. Microarray analysis revealed that several miRNAs, including the let-7 family (let-7a to i), display a striking upregulation in differentiating SK-3^{rd} cells and a high level of expression in the parental SK-BR-3 cells that have not been enriched for breast T-ICs[13] (Table 1). These findings are similar to that of let-7b and let-7c being increased in differentiated Comma-Dβ cells.[13] As discussed in the section "Differential miRNA Expression in Breast Cancer", let-7–related miRNAs were generally

Table 1 An Abridged List of Differentially Expressed miRNAs in Breast Cancer and Their Known Functions in Normal or Neoplastic Tissue

miRNA	Expression in Breast Cancer	Known Functions in Normal and/or Neoplastic Tissue
let-7 family	↓ NC tumor; ↑ Lum B, ↓ Her2+ ↑ PR+ vs. PR– ↓ ERα– vs. ER+	Targets HMGA2,[18] RAS[17]; downregulated in malignancies (introduction of let-7 induces cell death and cell-cycle arrest in lung carcinoma[75]); breast cancer stem cell maintenance[16]
miR-15/miR-16	↓ NC tumor	Deleted in chronic lymphocytic leukemia; targets BCL-2[76]
miR-17–5p	↑ NC tumor	Targets tumor suppressor Rbl2; targets AIB1[37]; overexpression promotes proliferation[77] and antagomiRs induce apoptosis in lung cancer cells[78]
miR-18a	↑ BL; ↓ Lum A	Overexpression accelerates tumor development and cell proliferation[79,80]
miR-21	↓ Norm; ↑ NC tumor; ↑ BL	Targets tumor suppressor genes TPM1, PDCD4, and maspin; inhibition results in decreased cell growth and metastasis[50,51,69]
miR-26a	↑ Norm; ↑ Lum A; ↓ Her2+ ↓ ERα– vs. ER+ ↑ PR+ vs. PR–	Targets SMAD1 transcription factor and modulates osteoblast differentiation[81]; promotes myogenesis by targeting a suppressor of muscle differentiation[82]
miR-29	↑ NC tumor; ↓ Lum A; ↓ Her2+	Downregulated in B-cell malignancies and colorectal cancers; targets ERK5; overexpression inhibits cell growth[83]; overexpression in adipocytes represses insulin-stimulated glucose uptake and causes insulin resistance[84]
miR-106a	↓ Norm; ↑ BL; ↓ Lum A; ↑ Lum B	Overexpressed in T-cell leukemia; oncogenic potential in anchorage-independent assay[85]
miR-122a	↑ NC tumor	Targets cyclin G1[86] and CT-1 amino acid transporter[87]; regulates lipid metabolism[88]; facilitates replication of HCV in liver[89]
miR-126	↑ Norm; ↓ Lum B	Potential metastasis suppressor in human breast cancer[35]
miR-130a	↑ Norm; ↓ NC tumor; ↓ Her2+	Targets homeobox genes GAX and HOXA5; antagonizes their antiangiogenic activity[90]
miR-143/miR-145	↓ NC tumor; ↓ BL; ↓ Lum A; ↓ Her2+ ↓ ErbB2– vs. ErbB2+	Decreased in B-cell malignancies, colorectal cancer, breast cancer; targets ERK5; overexpression inhibits cell growth in Raji cells[91]
miR-146	↑ NC tumor	Endotoxin responsive; NF-κB dependent; involved in TLR and cytokine signaling in innate immune response[92]
miR-150	↓ Lum B; ↑ Her2+ ↑ ERα– vs. ER+	Controls B-cell differentiation by targeting c-Myb[93]
miR-155	↑ NC tumor; ↓ Lum B	Overexpressed in B-cell lymphomas[94]; targets TP53INP1 (proapoptotic stress-induced p53 target gene) in pancreatic ductal carcinoma[95]
miR-181a	↑ NC tumor	Involved in modulation of T-cell sensitivity and selection[96]
miR-200b	↓ Norm; ↑ Her2+	Overexpressed in malignant cholangiocytes; inhibition increases sensitivity to gemcitabine[97]
miR-200c	↓ Norm; ↓ Lum A	Targets TCF8; ectopic expression in breast cancer cells restores E-cadherin expression and alters cell morphology[71]
miR-206	↑ NC tumor ↑ ERα– vs. ER+	Targets ERα in breast cancer cells; expression modulated by 17β-estradiol[33] regulates muscle development[36]
miR-214	↓ Norm; ↓ Lum A	Targets PTEN; induces cell survival and cisplatin resistance[98]
miR-221/miR-222	↑ BL; ↓ Lum A; ↓ Her2+	Targets cell-cycle regulator p27 (Kip1), mediates cancer cell growth,[99] erythropoiesis[100]
miR-224	↓ NC tumor; ↓ Her2+	Targets apoptosis-inhibitor 5 (API-5); increases apoptotic cell death[101]

Norm, normal/normal-like; NC tumor, nonclassified tumor; BL, basal-like; Lum A, luminal A; Lum B, luminal B.

decreased in breast cancer versus normal tissue or in more transformed/aggressive versus less transformed breast cancers (Table 1). Collectively, these studies raise the possibility that silencing of let-7 expression may contribute to the "stemness" in breast T-ICs. Support for this hypothesis was gained through functional studies by Yu et al[16] Overexpression of let-7a in SK-3rd cells and in primary patient breast T-ICs reduced mammosphere formation and reduced tumor formation and metastases in transplants of SK-3rd cells into mice. Experimental

elevation of let-7a both reduced the burst of proliferation as SK-3rd cells entered a differentiation pathway and reduced the number of undifferentiated cells after 10 days of differentiation-inducing culture conditions. Two cellular oncogenes, RAS and HMGA2, are targets of let-7.[17,18] In nonenriched, parental SK-BR-3 cells, H-RAS and HMGA2 protein levels were low, and knockdown of let-7 increased both proteins.[16] In T-IC-enriched SK-3rd mammospheres, H-RAS and HMGA2 levels were elevated, and were decreased by forced expression of let-7. In summary, this study[16] supports a functional role of let-7–related miRNAs in the loss of the ability of self-renewal in T-ICs, as well as promotion of differentiation into luminal and basal cell types. It is worth noting that this study did not identify a miRNA that is highly expressed in the SK-3rd mammosphere cells and markedly downregulated with differentiation (i.e., a miRNA that potentially promotes "stemness").

DIFFERENTIAL miRNA EXPRESSION IN BREAST CANCER

The first link between miRNA expression and cancer was provided by Calin and colleagues,[19] who reported that the miR15a/16–1 cluster was deleted in the majority of chronic lymphocytic leukemia (CLL) cases. Calin and colleagues also reported that many cancer-associated and fragile chromosomal sites encompass miRNA genes.[20] Similarly, Zhang and colleagues[21] reported that a large percentage of miRNA genes show loss or gain of copy number in three cancer types, including breast, as well as a gain of copy number of the miRNA-processing enzymes, Dicer and Argonaute-2, in ovarian cancer. These studies established genomic alteration as one cause of differential expression in normal versus breast cancer tissues.

Results from miRNA profiling across multiple human cancers indicated that miRNAs were potent indicators of developmental origin, or, in poorly differentiated cancers, the cell type of origin.[22] The clear cell-specific expression of miRNA genes was further emphasized by Jiang et al.[23] In 2005, the Croce laboratory reported the differential expression of miRNAs among human breast cancers.[24] Using microarray analysis of miRNA expression in 76 individual samples of breast cancer and 10 pooled normal samples, Iorio et al reported 29 miRNAs (or ~12% of the of 245 human and mouse miRNAs represented on the array) that showed differential expression between cancerous and normal samples.[24] Of note, miR-10b, miR-125b, and miR-145 were significantly downregulated in the majority of breast cancer samples and breast cancer cell lines, whereas miR-21 and miR-155 were strongly upregulated (Table 1). Thus, these findings indicated that both oncogenic and tumor suppressor miRNAs might be dysregulated during oncogenic progression. In the Iorio et al study,[24] associations between differentially expressed miRNAs and several clinicopathologic features, including hormone receptor expression, were also reported. This study probably raised more questions than answers. For example:

1. What is the molecular basis (e.g., chromosomal deletion, epigenetic changes, etc.) for the altered expression of a given miRNA?
2. What degree of altered expression of a specific miRNA is required to effect a change in the biology of the cell?
3. What are the mRNA targets of the differentially expressed miRNAs?
4. Is there a causal relationship between a differentially expressed miRNA and any aspect of neoplastic transformation?

Nevertheless, this seminal study raised the very real possibilities that aberrant miRNA expression might contribute to the oncogenic process in breast cancer and that miRNA signatures may have diagnostic value in this heterogeneous form of cancer.

A subsequent study from the Croce laboratory that addressed differentially expressed miRNAs in breast and five other solid tumors (colon, lung, pancreas, stomach, prostate) showed that 36 miRNAs were upregulated in cancers and 21 were downregulated.[25] Notably, miR-21 was upregulated in all six tumor types, and miR-155 was upregulated in breast, colon, and lung tumors. Four other miRNAs, miR-17–5p, miR-29b–2, miR-181b–1, and miR-146 were upregulated in breast and at least two other tumor types (Table 1).

Mattie and colleagues[26] used a unique microarray assay of nanogram quantities of miRNA-enriched samples from prostate and breast tumors, which revealed a subset of miRNAs (let-7f, let-7 g, miR-107, miR-10b, miR-126, miR-154, and miR-195) whose expression was higher in ErbB2/HER2-negative versus ErbB2/HER2-positive cancers (Table 1). Additionally, a subset of miRNAs (miR-142–5p, miR-201, miR-205, and miR-25) showed higher expression in ERα/progesterone receptor (PR)-positive versus ERα/PR-negative cancers (Table 1). As first suggested by Iorio et al,[24] these results indicated that miRNA expression might modulate hormone receptor expression (see next section), which has a significant impact on treatment and prognosis of breast cancers.

In recent years, microarray analyses of protein-encoding gene expression among different breast cancers have demonstrated the existence of fundamentally different types of cancer.[2,3] These molecular analyses have revealed the presence of at least four subtypes, based on luminal or basal markers (e.g., cytokeratins and cell-cell and cell-matrix adhesion proteins) and on the expression of steroid hormone receptors and/or ErbB2/HER2 (hitherto referred to as HER2). The *luminal A* subtype,

which is the most commonly diagnosed form of breast cancer, is typically of low grade (grades 1 or 2), is ERα-positive, PR-positive, HER2-negative, and responds well to selective estrogen receptor modulators (SERMs) (e.g., tamoxifen) and aromatase inhibitors and has a good prognosis. A subset of luminal A cancers can be more precisely categorized as *luminal B*, which respond to SERMs and aromatase inhibitors but have higher levels of proliferative indices (e.g., K_i-67 expression), are of a higher grade (grades 2 and 3), and have a significantly lower frequency of relapse-free survival than do luminal A cancers. A third subset is *basal-like*, which expresses basal-specific markers (see above) and, consistent with the basal position of myoepithelial cells in the mammary epithelium, may display myoepithelial characteristics such as smooth muscle α-actin. The basal-like subtype is typically a high-grade tumor (grade 3) that does not express ERα, PR, or HER2, although a significant percentage overexpresses the epidermal growth factor receptor (EGFR).[27] Basal-like tumors are responsive to chemotherapy, as opposed to current "hormonal" therapies (although EGFR-positive cancers may be found to respond to EGFR-selective tyrosine kinase inhibitors). The basal-like subset is associated with breast cancers that develop in women harboring a BRCA1 mutation[3] and, in general, has a higher risk of recurrence than do luminal A cancers. The fourth subset of breast cancer, the *HER2* subset, display a robust expression of HER2, usually due to gene amplification. These cancers are of a high grade (grade 3), occur at an earlier age than do other subtypes, do not express ERα or PR, and have a lower probability of relapse-free survival than do luminal A cancers. This subset of patients can respond to anti-HER2 therapies (e.g., trastuzumab) in conjunction with chemotherapy. Other less well-characterized subsets have also emerged, namely *normal-like* (good prognosis) and *interferon-regulated genes* (poor prognosis).[2]

Blenkiron and colleagues[28] examined whether miRNA expression profiles correlate with the mRNA expression-based classification scheme described above. Using array technology to detect 309 human miRNAs, this group analyzed 99 primary tumors, 5 normal breast samples, and 33 breast cancer cell lines. These investigators observed that miRNAs were differentially expressed in a manner that allowed them to be used in the subtype classification scheme described above. For example, 10 miRNAs were significantly higher and 15 miRNAs were significantly lower in ERα-negative versus ERα-positive tumors. Whereas this profile correlated with grade, it was inversely correlative between the basal and luminal A subtypes (Table 1). Luminal B and HER2 showed a lesser degree of correlation. However, the entire picture of differentially expressed miRNAs was sufficient to assign the five subtypes described above. These investigators gained evidence for a complex set of factors involved in the dysregulation of specific miRNAs, including genomic alterations, transcriptional regulation, and posttranscriptional regulation/processing of miRNAs.

Blenkiron et al[28] also reported that the levels of two miRNA processing/effector enzymes showed trends accordingly to subtype and ERα expression. Argonaute-2 (Ago2) and Dicer1 were expressed at higher levels in the more transformed subtypes (basal-like, HER2, and luminal B) relative to luminal A and normal-like tumors. There is currently very little known about the regulation of the expression of miRNA processing and effector enzymes, including their expression and regulation in breast cancer cells. Consistent with these findings,[28] our laboratory has observed elevated levels of Ago2 mRNA and protein in two ERα-negative human breast cancer cell lines (MDA-MB-231, MDA-MB-435) compared with that in two ERα-positive cell lines (MCF-7, T47D). Additionally, these high levels of Ago2 expression are dependent on the mitogen activated kinase pathway (Adams BD and White BA, unpublished data). It remains to be determined whether changes in the capacity of the miRNA processing/effector apparatus alter the efficacy of miRNA-mediated suppression of gene expression either globally or selectively.

One significant limitation of microarray, quantitative RT-PCR, and Northern blot analyses of RNA samples is the lack of information with respect to single cell-type expression of miRNAs within a heterogeneous tissue sample. Sempere and colleagues[14] used locked nucleic acid (LNA) probes (which form highly stable hybrids and thereby afford increased sensitivity, stringency, and specificity) in both microarrays and in situ hybridization assays. Using microarray analysis of matched normal/tumor RNA samples, these investigators reported that, in general, miRNAs were upregulated in tumor versus normal samples. Not surprisingly, miR-21 was consistently upregulated in tumor versus normal samples (Table 1). The authors note that miR-205 was elevated in two of four of the ERα/PR/HER2-negative specimens, perhaps suggesting that miR-205 is important in the progression of basal-like tumors (Table 1). Nontumorigenic (immortalized) and tumorigenic cell lines were also examined by microarray and Northern blot and revealed that miR-21, miR-23, and miR-191 were elevated in the tumorigenic cell lines (Table 1). Eleven of the breast cancer cell lines were able to be classified as basal or luminal based on mRNA profiling and cytokeratin expression. In basal cell lines, miR-221 and miR-222, which are linked on chromosome X, display basal-specific expression. In contrast, miR-141 and miR-200c, linked on chromosome 12, and miR-183 display an almost entirely luminal-specific expression.

Sempere et al[14] also used the LNA probes for in situ hybridization to examine cell-specific miRNA expression. Let-7a was expressed primarily in luminal epithelial cells and was significantly decreased in tumor cells versus matching normal epithelial cells, a finding

that may be related to the downregulation of let-7–related miRNAs in differentiated stem and T-IC cells (Table 1). miR-21 was expressed in luminal cells and was increased in tumor versus normal cells, and miR-145 was expressed in myoepithelial cells throughout ducts and lobules and in vascular smooth muscle cells (Table 1). This miRNA was detected primarily in the nucleus, raising the possibility of a nuclear function of this miRNA. Because the in situ hybridization probe would not distinguish between mature and precursor miRNA, this could also be explained by a precursor that remains in the nucleus, as recently described for some miRNA precursors.[29] Expression of miR-145 decreased in hyperplastic samples and was very low or absent in carcinoma in situ and invasive carcinoma samples (Table 1). As mentioned above, miR-205 levels were found to be elevated in two of four basal-like cancer specimens. In situ hybridization of more samples revealed that miR-205 was expressed in normal myoepithelial cells and, for the most part, decreased in cytokeratin 14–positive (i.e., basal-like) carcinoma cells. The authors note that low expression of miR-205 in basal-like (ERα/PR/HER2-negative) breast cancer is predictive of a favorable clinical outcome, whereas high miR-205 is associated with a poor outcome. Although more samples need to be tested, miR-205 may prove to be a novel marker that distinguishes between two subtypes of basal-like breast cancers. Finally, miR-451, which was downregulated in breast tumor specimens, was only expressed in erythrocytes. Thus, in situ hybridization can distinguish between epithelial-derived carcinoma and stromal expression of miRNAs, thereby providing greater insight into identifying differentially expressed miRNA (in this case, decreased expression of miR-451 was probably due to poor vascularization of the tumor core).

INVOLVEMENT OF miRNAs IN THE HORMONAL AND GROWTH FACTOR SIGNALING PATHWAYS IN BREAST CANCER

Involvement of miRNAs in Estrogen Signaling Pathways

Breast development in women primarily occurs peripubertally and postpubertally in response to ovarian, placental, and pituitary hormones. Both stromal and epithelial cells respond to systemic hormones and interact with each other through the release of paracrine growth factors.[30] In normal human mammary epithelium, luminal cells proliferate at a much higher rate than do basal myoepithelial cells.[31] Current evidence indicates that among all basal and luminal epithelial cells lining the mammary tree, only a small subpopulation of normal luminal cells, and no basal myoepithelial cells, express ERα.[31] Moreover, current models indicate that 17β-estradiol stimulates normal ductal cell proliferation indirectly through the secretion of a paracrine factor (e.g., amphiregulin), which stimulates proliferation of adjacent ERα-negative cells.

Although the percentage of ERα-positive cells increases with age, the relative paucity of ERα distribution within the luminal mammary epithelial cells is in marked contrast with the fact that about two thirds of initially diagnosed breast cancers display some degree of ERα expression in postmenopausal women.[31] Indeed, most newly diagnosed breast cancers fall into the luminal A subtype and respond well to antiestrogen/antiaromatase therapies. The basis for the predominance of ERα-positive tumors is poorly understood but is very likely to be related to the proliferative and antiapoptotic effects of an enriched local environment of 17β-estradiol,[32] possibly during selection of T-IC–derived ERα-positive and -negative "progenitor cancer" cells. In any case, the molecular basis for the appearance of ERα/PR-positive cancers, which have a better prognosis, as opposed to the appearance of ERα/PR-negative basal-like and HER2 cancers, which have a poorer prognosis, is an important goal of breast cancer research.

To gain more insight into the mechanisms that underlie the initiation and progression of ERα-positive breast cancers, many groups have studied the regulation of ERα gene expression. Regulation of the expression of the human ERα gene (ESR1) is complex and can occur at the transcriptional, posttranscriptional, and posttranslational levels.[33] Posttranscriptional regulation of ERα mRNA includes targeting by miRNAs. Adams et al[33] reported that the relatively long 3′ UTR of the human ERα mRNA is enriched in putative miRNA target sites. This is consistent with findings that mRNAs encoding dynamic, regulatory proteins, as opposed to "housekeeping" proteins, have evolved longer 3′ UTRs enriched with miRNA target sites.[34] Adams et al reasoned that miRNAs that were increased in ERα-negative versus ERα-positive breast cancers (Table 1), as described by Iorio et al,[24] might play a role in downregulation of ERα expression. Of three such miRNAs, miR-206 was identified as having two putative target sites within the ERα mRNA 3′ UTR. Overexpression of miR-206 repressed endogenous ERα mRNA and protein in MCF-7 cells. Also, the two putative miR-206 target sites conferred miR-206–specific suppression in a heterologous luciferase 3′ UTR reporter construct. Differential upregulation of miR-206 expression has not yet emerged as a marker of ERα-negative, basal-like, or HER2 subsets in recent studies (Table 1). However, it is noteworthy that miR-206 was one of the eight miRNAs whose level was decreased in metastasis-derived cells detected by Tavazoie et al,[35] and that among the 20 breast tumors assayed in this study, 9 of 10 tumors that showed lower than average miR-206 expression were ERα-positive, whereas 7 of 10 tumors that showed higher than average

miR-206 expression were ERα-negative. miR-206 remains of interest for several reasons. First, Adams et al[33] reported that, in a negative feedback loop, 17β-estradiol and the ERα-selective agonist, PPT, repressed miR-206 expression. Thus, ERα and miR-206 may interact in a manner that confers bistability on ERα-positive (e.g., luminal) versus ERα-negative (e.g., luminal or myoepithelial) phenotypes (Fig. 1). Also, overexpression of

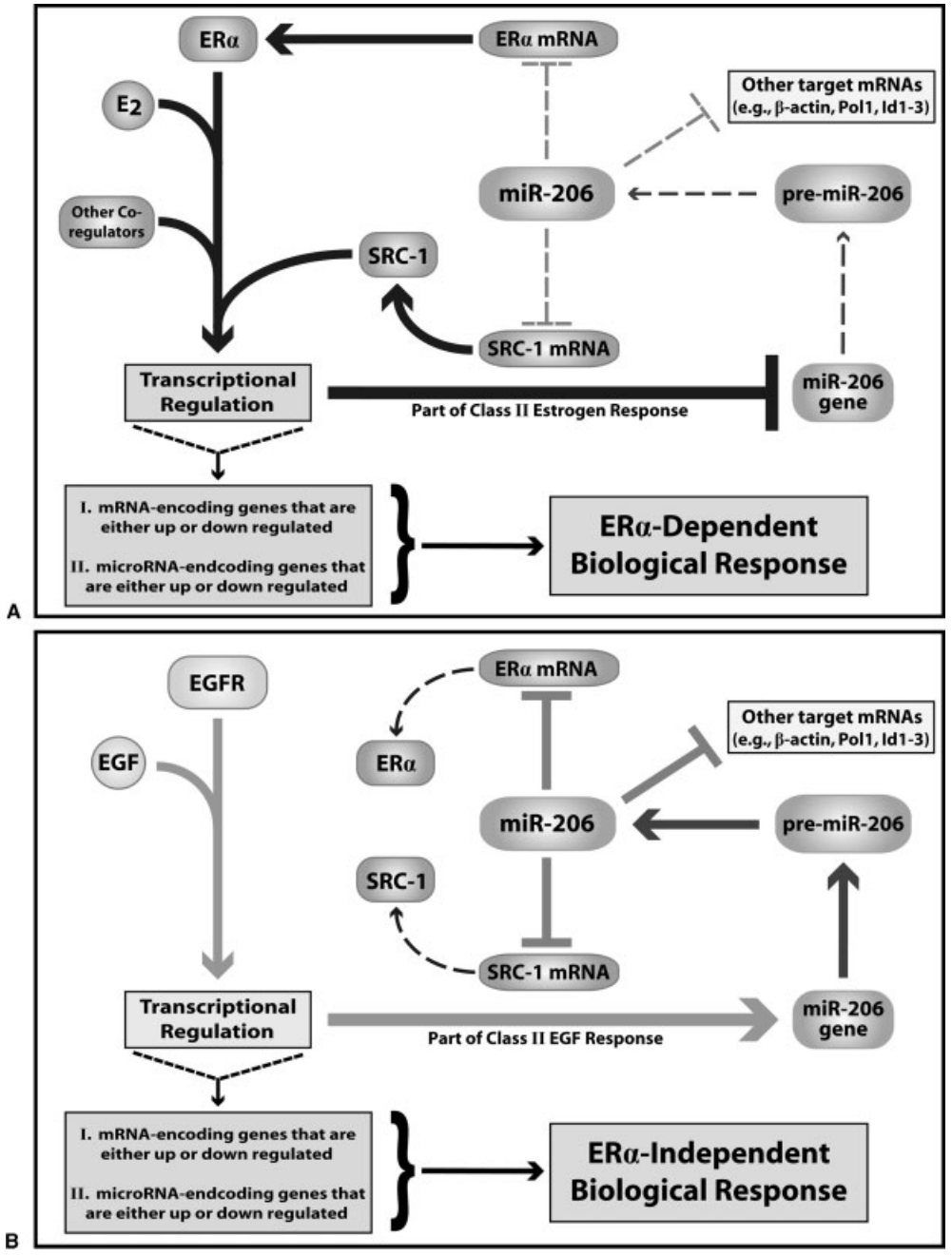

Figure 1 Bistability of ERα-positive versus ERα-negative phenotypes in breast cancer cells: proposed interactions among ERα, EGFR, and miR-206. (A) In ERα-positive breast cancer cells, 17β-estradiol promotes growth and survival through the ERα signaling pathway, which involves the transcriptional regulation of mRNA/protein-encoding genes (I) and miRNA-encoding genes (II). Active ERα signaling inhibits miR-206, which in turn targets ERα and SRC-1[33] (Adams BD and White BA, unpublished data). Thus, ERα signaling maintains ERα expression through the repression of an inhibitory signal. (B) EGF signaling is antagonistic of ERα activity and evokes a biological response in part by regulation of mRNA/protein-encoding genes (I) and miRNA encoding genes (II). EGFR signaling strongly upregulates miR-206 levels (Adams BD and White BA, unpublished data). We propose that EGFR-induced miR-206 expression contributes to the posttranscriptional silencing of ERα and SRC-1 gene expression in EGFR-positive cancers, especially basal-like. Increased miR-206 also targets other mRNAs (see inset and Table 1), which may contribute to a *myoepithelial-like* phenotype.

miR-206 strongly reduced endogenous levels of nonmuscle β-actin in MCF-7 cells,[33] consistent with other reports that miR-206 stimulates myogenic differentiation.[36] Thus, miR-206 may play a role in the normal differentiation of ERα-negative myoepithelial cells and, as such, be expressed in a highly cell-specific manner. Recent studies have also shown that miR-206 targets the mRNAs encoding two p160 family coregulators of ERα: steroid receptor coactivator-1 (SRC-1) and SRC-3 (also called augmented in breast cancer 1, or AIB1) (Adams BD and White BA, unpublished data). Thus, miR-206 appears to coordinately suppress multiple components of the 17β-estradiol/ERα signaling pathway, a feature that would be expected to confer greater stability of ERα-negative myoepithelial differentiation. Our laboratory has also observed that epidermal growth factor (EGF) stimulates miR-206 expression in ERα-positive MCF-7 and ERα-negative MDA-MB-231 cells (Adams BD and White BA, unpublished data). This is interesting in light of the finding that the EGFR expression is frequently upregulated in basal-like breast cancer cells with myoepithelial-like differentiation[27] and suggests one mechanism by which EGF signaling could maintain an ERα-negative phenotype (Fig. 1).

Hossain and colleagues[37] demonstrated that the ERα-coactivator, AIB1 (SRC-3), is also targeted by the miRNA miR-17–5p. In this study, miR-17–5p inhibited translation of ERα mRNA without affecting its levels. AIB1 is amplified or overexpressed in up to 60% of human breast cancers and has been clearly demonstrated to be oncogenic in transgenic mouse models.[38] AIB1 acts as a coactivator for both ERα and the cell-cycle–related transcription factor, E2F1. Hossain et al[37] showed that overexpression of miR-17–5p downregulated the transactivating activity of ERα and E2F1 proteins and decreased the target genes of these transcription factors. The level of miR-17–5p was very low in breast cancer cell lines (compared with nontransformed mammary epithelial cell lines; Table 1). Overexpression of miR-17–5p suppressed both estrogen-dependent and independent proliferation of MCF-7 and BT20 breast cancer cell lines, respectively. MiR-17–5p also suppressed IGF-1–induced, anchorage-independent growth of MCF-7 cells. These findings indicate that miR-17–5p acts as a tumor suppressor in breast cancer, in part, by inhibiting expression of AIB1, resulting in decreased transcriptional activity of ERα and E2F-1.[37]

Several studies have revealed the existence of a complex feed-forward circuitry among c-Myc, E2F proteins and specific miRNAs encoded by the miR-17/92 cluster (which includes miR-17–5p).[39] Woods and colleagues showed that E2F1, E2F2, and, in particular, E2F3 directly stimulated transcription of the miR-17/92 cluster.[40] In an accompanying paper, Sylvestre and colleagues[41] also observed direct regulation of the miR-17/92 cluster by E2F proteins. They further demonstrated that miR-20a (from this cluster) exerts translational repression on the 3′ UTRs from E2F1, E2F2, and E2F3, especially the E2F1 3′ UTR. As E2F1 has been shown to promote apoptosis in response to certain stresses,[42] this group examined whether miR-20a altered the cellular response to a stress. Experimental reduction and augmentation of miR-20a expression increased and inhibited, respectively, the ability of doxorubicin to induce apoptosis in prostatic PC3 cells. These findings indicated that miR-20a acted as an antiapoptotic miRNA that could promote tumor progression.

In an attempt to put these studies in the context of estrogen-responsive breast cancer, underexpression of the miR-17/92 complex in breast cancer would allow for robust expression of AIB1 and thus enhanced ERα and E2F1 transcriptional activity. Although elevated levels of E2F1, due to low miR-20a expression, might expose the cells to DNA damage–induced apoptosis,[42] this could be negated by the antiapoptotic effects of strong estrogen signaling.[43] It is worth noting that in a cluster of 51 breast cancers that could be classified according to subtypes (luminal A, etc.; see above), the highest levels of miR-17–5p expression occurred primarily in ERα-negative, basal-like tumors (Table 1).[28]

miRNAs have also been implicated in the regulation of steroid hormone metabolism. The enzyme CYP1B1 is a member of the cytochrome P450 superfamily of enzymes and has been shown to be overexpressed in several human cancers, including invasive ductal carcinomas of the breast.[44] CYP1B1 catalyzes the activation of several procarcinogens and also converts estradiol into 4-hydroxyestradiol (a catechol-estrogen), which generates free radicals that cause DNA damage. Unlike CYP1B1 protein levels, CYP1B1 mRNA levels do not significantly differ between tumor and normal tissues.[45] This discrepancy prompted Tsuchiya and colleagues[44] to examine whether CYP1B1 is regulated posttranscriptionally by miRNAs. In silico analysis revealed miR-27b as a potential regulator. Luciferase constructs harboring the miR-27b site in the 3′ UTR were repressed in MCF-7 breast cancer cells, which express readily detectable levels of miR-27b, but not in Jurkat cells expressing almost nondetectable levels of miR-27b. Forced expression of miR-27b in Jurkat cells repressed the expression of various miR-27b–containing luciferase constructs, whereas suppression of miR-27b in MCF-7 cells relieved repression of the same constructs. Experimental manipulation of miR-27b also altered, in an inverse manner, endogenous CYP1B1 protein and activity levels. Tsuchiya et al[44] reported a decreased level of miR-27b in breast cancer by comparing paired normal and cancerous breast specimens, as well as an inverse relationship between miR27b and CYP1B1 in breast cancers. The involvement of miRNAs in the regulation of other steroid hormone–modulatory enzymes, such as

various forms of 17β-hydroxysteroid dehydrogenases, steroid sulfotransferases and sulfatases, and CYP19 aromatase, has not been reported and clearly deserves further study in both normal and neoplastic steroid hormone–responsive organs.

ErbR/HER信号通路相关miRNAs

Involvement of miRNAs in ErbB/HER Signaling Pathways

Members of the ErbB/HER family of receptors, which is composed of EGFR, HER2, HER3, and HER4, are frequently amplified or overexpressed in breast cancer, especially in ERα-negative forms (see above). In two of the microarray studies described above,[24,26] miR-125b was underexpressed in breast cancer versus normal tissue, breast cancer cells versus noncancerous cells, and, in particular, in HER-positive versus HER2-negative breast cancers (Table 1). This prompted Scott et al[46] to examine whether miR-125b, and/or its homolog, miR-125a, targeted any of the HER receptors. In silico analysis revealed that the 3′ UTRs of HER2 and HER3 harbored putative miR-125 sites. Overexpression of miR-125a, and to a lesser extent miR-125b, led to a downregulation of HER2 and HER3 in human breast cancer SK-BR-3 cells (the effects of miR-125 on HER1 and HER4 were apparently not examined). Overexpression of miR-125a also decreased signaling through mitogen-activated protein kinase (MAPK) and AKT pathways. Scott et al[46] also demonstrated that miR-125a and miR-125b inhibited anchorage-dependent growth of HER2-dependent SK-BR-3 cells, but much less so in HER2-independent, nontransformed MCF-10A cells. Similarly, miR-125a and miR-125b inhibited SK-BR-3 cells, but not MCF-10A cells, in an in vitro migration assay and also inhibited SK-BR-3 cells in an in vitro invasion assay. This important study provides an impetus for future research on the targeting of a specific miRNA for the treatment of a subset of breast cancers (i.e., HER2).

Another aspect of ErbB/HER signaling that involves miRNA regulation is the interactions among MUC1, galectin-3, and EGFR. MUC-1 is mucin-related transmembrane glycoprotein that is normally limited to the apical surface of polarized epithelia, which extends into the luminal space well beyond the glycocalyx.[47] In several types of neoplasms, including breast cancer, normal cell-cell and cell-matrix adhesions are reduced or lost. Polarity is not maintained, and MUC-1, which is often overexpressed, gains access to the entire cell membrane, affording a protective barrier to many forms of extracellular stress. During synthesis and processing, MUC1 is proteolytically cleaved into an N-terminus extracellular domain and is noncovalently attached to the MUC1 C-terminus (MUC1 C), which is composed of a short extracellular domain, a transmembrane domain, and a cytoplasmic domain. EGF causes clustering of MUC1 and the EGFR, thereby recruiting MUC1 C to intracellular signaling molecules, including β-catenin.[47]

Ramasamy et al[47] recently described that MUC1 C inhibits expression of the miRNA miR-322, which targets galectin-3. In BT549 breast cancer cells, MUC1 and galectin-3 are weakly expressed, and forced expression of MUC1 increased galectin-3. Conversely, silencing of high endogenous levels of MUC1 expression in ZR-75–1 breast cancer cells led to a decrease in galectin-3. Further analyses with promoter/reporter constructs indicated that MUC1 C increases galectin-3 posttranscriptionally. In silico analyses identified miR-322 as a possible repressor of galectin-3 expression. Studies showed that miR-322 levels were repressed by a MUC1-dependent mechanism and that miR-322 represses the expression of galectin-3. This study also demonstrated that galectin-3 binds to the extracellular domain of N-glycosylated MUC1 and acts as a bridge between MUC1 and the EGFR in ZR-75–1 cells.[47] Because both MUC1 and galectin-3 are frequently overexpressed and have the ability to transform cells, this study has identified an apparently crucial linchpin, miR-322, in the positive feed-forward loop between MUC1 and galectin-3 and the ability of MUC1 to cluster with the EGFR and associate with multiple signaling components. Thus, future research may show that upregulation of miR-322 levels, along with EGFR-selective tyrosine kinase inhibitors, may prove effective in the treatment of breast cancers.

The importance of miR-322 may extend well beyond EGFR signaling and breast cancers in which the EGFR is overexpressed (e.g., basal-like tumors with myoepithelial differentiation). Wei and colleagues have also demonstrated that MUC1 C also interacts with and stabilizes ERα in MCF-7 breast cancer cells.[48] Further, chromatin immunoprecipitation assays showed that MUC1 C increases the recruitment of the p160-family coactivator proteins SRC-1 and GRIP-1 to promoter-bound ERα. Thus, overexpression of miR-322 might be an effective treatment for luminal-type as well as basal-type breast cancers.

INVOLVEMENT OF miRNAs IN CELL SURVIVAL AND CELL-CYCLE TRAVERSE IN BREAST CANCER

miR-21 is overexpressed in breast cancer, specifically in luminal epithelial cells, which has been confirmed by several independent studies (Table 1). This prompted Si et al[49] to examine the effects of miR-21 knockdown in MCF-7 breast cancer cells. Anti-miR-21 both suppressed basal proliferation of MCF-7 cells and sensitized the cells to an anticancer drug, topotecan. Transfection of MCF-7 cells with anti-miR-21, followed by xenografting into the mammary pads of female nude mice, resulted in smaller tumors (as opposed to ones

transfected with a "negative control") after 28 days. Anti-miR-21 also promoted apoptotic death in MCF-7 cells in vitro.[49] This group subsequently used a proteomic approach to the search for miR-21 targets by directly injecting anti-miR-21 or a negative control into xenografted MCF-7–derived, 17β-estradiol–supported tumors in the mammary pads of nude female mice.[50] After 4 weeks, tumors were harvested and analyzed by two-dimensional differentiation in-gel electrophoresis and mass spectrometry. Though the levels of several proteins were increased by anti-miR-21 injections, this group focused on the tumor suppressor, tropomyosin 1 (TPM1). A putative miR-21 binding site was identified in the variant 1 TPM1 transcript, and subsequent studies showed that this site conferred miR-21–induced regulation of luciferase and green fluorescent protein (GFP) reporter constructs.[50] Moreover, experimental manipulation of miR-21 levels induced a reciprocal change in the levels of TPM1 protein but not TPM1 mRNA.

The role of miR-21 in the regulation of apoptosis was further addressed by Frankel et al,[51] who searched for targets of miR-21 in MCF-7 cells by microarray analysis of mRNA expression after transfection with a LNA anti-miR-21 (or scrambled) oligonucleotide. Remarkably, inhibition of miR-21 for 24 hours resulted in the upregulation and the downregulation of 402 and 335 mRNAs, respectively. Analysis of these transcripts identified several survival-related gene ontologies. First, several p53-regulated transcripts, including FAM3C, ACTA2, APAF1, BTG2, FAS, CDKN1A (p21), and SESN1, were upregulated by anti-miR-21 treatment.[51] Although p53 itself was not targeted by miR-21, p53 knockdown studies in the presence of anti-miR-21 treatment indicated that miR-21 antagonizes p53 function by targeting several of its downstream effectors. Given the recent findings that miR-34a is a downstream effector of p53 in ovarian, lung, and colon cancer cells,[52–56] it will be of interest to examine whether miR-21 expression is reciprocal to that of miR-34a in breast cancers. This study also identified the mRNA encoding programmed cell death 4 protein (PDCD4) as a direct target of miR-21 in MCF-7 cells.[51] PDCD4 has been characterized as a tumor suppressor.[57] Knock-down of PDCD4 partially blocked the antiproliferative effect of anti-miR-21 treatment in MCF-7 cells.[51] The regulation of PDCD4 by miR-21 was also observed by Asangani et al[58] in colorectal cancer cell lines. These authors also found an inverse correlation between miR-21 and Pdcd-4 in 22 paired normal/tumor colorectal cancers (Table 1). Importantly, Asangani et al also reported that overexpression of miR-21 increased invasive activity of colon cancer cells in vitro, and that knock-down of miR-21 inhibited intravasation and lung metastasis of colon cancer cells in a chick embryo metastasis assay.[58] This prometastatic aspect of miR-21 has recently been examined in breast cancer cells (see next section).

Sp transcription factors are among the numerous checkpoint proteins that regulate cell-cycle traverse and are often overexpressed in several cancers, including breast cancer.[59] In a study of the regulation of miRNAs and mRNAs by histone deacetylation in the SK-BR-3 breast cancer cells, Scott and colleagues[60] observed that 22 miRNAs were downregulated and 5 miRNAs were upregulated after a 5-hour treatment with the histone deacetylase (HDAC) inhibitor LAQ824. Of these, subsequent study focused on miR-27a and miR-27b. Of the predicted targets for miR-27a and miR-27b, the transcription factor, ZBTB10/RINZF, was reproducibly upregulated by knock-down of miR-27a and miR-27b.[60] As ZBTB10 is an antagonist of Sp1, these findings by Scott et al[60] prompted Mertens-Talcott and colleagues[61] to test the hypothesis that upregulation of Sp protein expression or activity in breast cancer may be regulated by miR-27a or miR-27b. These researchers confirmed that ZBTB10 levels are increased by knock-down of miR-27a in MDA-MB-231 cells, indicating that ZBTB10 mRNA is probably targeted by miR-27a, although further work is needed to directly confirm the miR-27a target site. Interestingly, knock-down of miR-27a also led to a downregulation of Sp1, Sp3, and Sp4 mRNA and protein levels, and further examination revealed that miR-27a repressed transcriptional activity of Sp1 and Sp3 promoters within heterologous promoter/luciferase constructs.[61] Overexpression of ZBTB10 also repressed Sp protein gene expression in this study, and the authors reasonably concluded that miR-27a and miR-27b increase Sp1 gene expression through the repression of ZBTB10. This study also carefully examined the effects of miR-27a knock-down on cell-cycle traverse.[61] Experimental reduction of miR-27a increased the percentage of cells in G2/M phase, with no effect on the percentage in G0/G1. This is in contrast with the increase in the percentage of cells in G0/G1 observed upon overexpression of ZBTB10 or knockdown of Sp1. These results indicated that another miR-27a target was increased by miR-27a knockdown and arrested cells in G2/M phase. Further work revealed that the kinase, Myt-1, which inhibits cdc2, is upregulated by anti-miR-27a treatment.[61]

INVOLVEMENT OF miRNAs IN METASTASIS IN BREAST CANCER

Metastasis represents a complex series of molecular events involving the escape of tumorigenic cells from their primary location, their survival along the journey into, through, and out of vascular components or along the lining of a cavity (e.g., pleura, peritoneum), and their invasion into a new organ, followed by their survival and proliferation within a new tumor niche.[62–64] Recent

studies have revealed that miRNAs are involved in the orchestration of the events throughout this multistep process.

Ma et al[65] approached the identification of miRNAs that are involved in metastasis by revisiting the 29 miRNAs differentially expressed in breast cancer as described by Iorio et al.[24] Their research became focused on miR-10b, which was found to be expressed in the metastatic SUM1315 and MDA-MB-231 breast cancer cell lines. However, miR-10b was not expressed in the nontumorigenic HMEC and MCF-10A cell lines nor in the nonmetastatic SUM149, SUM159, and MCF-7 cell lines. Experimental manipulation of miR-10b levels demonstrated a positive relationship between miR-10b expression, migration, and invasion, as assayed in vitro. The ability of miR-10b to promote metastasis in vivo was addressed by implantation of miR-10b or mock-infected noninvasive, nonmetastatic SUM149 cells into mammary fat pads of immunodeficient mice. The miR-10b–overexpressing cells displayed an interesting sequence of events: at 6 weeks, no difference was observed in tumor size. However, the miR-10b cells showed invasion of surrounding connective tissue, underlying muscle, and vasculature. Additionally, staining the tumors with the proliferation marker K_i-67 and the endothelial marker MECA-32 indicated that miR-10b tumors displayed clear invasive fronts of K_i-67–enriched cells at the periphery of the tumor mass. Also, the miR-10b tumors were more vascularized, with vessels both in surrounding stroma and within the tumor itself. At 11 weeks, the miR-10b–overexpressing tumors were larger than controls, and this was attributed to more extensive invasive activity and better vascularization, as opposed to a higher rate of proliferation. At 9 and 11 weeks, lung metastases were observed in mice bearing either miR-10b SUM149 cells or miR-10b–infected invasive but nonmetastatic SUM159 cells.[24]

The Weinberg laboratory had previously shown that the transcription factor, Twist, promoted metastasis.[66] Ma et al[65] observed that differences in Twist expression correlated with miR-10b levels in cell lines with varying degrees of metastatic potential. Further work demonstrated that Twist increased miR-10b expression, probably by direct transcriptional activation. miR-10b, in turn, directly targeted the antimetastatic transcription factor HOXD10. Overexpression of HOXD10 without its 3′ UTR effectively blocked the ability of miR-10b to increase migratory and invasive activity SUM149 cells. Finally, the repression of HOXD10 by miR-10b led to an increase in the expression of the small Rho-family GTPase, RhoC, and knock-down of RhoC strongly antagonized the effects of miR-10b on migration and invasion. The relevance of miR-10b to human cancer metastasis was supported by the finding that miR-10b was normal in 5 of 5 metastases-free breast cancers, whereas miR-10b levels were elevated in 9 of 18 metastatic-positive breast cancers.[65]

Huang and colleagues used a different approach, a forward genetic screen, to examine the potential role of miRNAs in breast cancer metastasis. The Agami laboratory had previously cloned all annotated human miRNAs into a retroviral expression construct (individually called miR-Vecs, constituting a library, called miR-Lib) and had used a similar screen to implicate miR-272 and miR-273 in testicular cancer.[67] Huang et al[68] transduced nonmetastatic MCF-7 cells with ~450 individual miR-Vecs and then screened cells for migration in vitro. Identification of enriched miRNAs (as miR-Vecs) in the migratory subpopulation of MCF-7, compared with the levels in the total population, indicated that miR-373, miR-520c, and miR-520e were enriched in migratory cells. Stable overexpression of miR-373 and miR-520c produced highly migratory and invasive phenotypes, as assayed in vitro, without affecting cell proliferation. Endogenous expression of miR-373, but not miR-520, was detected in three migratory cancer cell lines, and knock-down of miR-373 abrogated the migratory phenotype of these cells. Huang et al[68] also showed that luciferase-tagged MCF-7 cells overexpressing either miR-373 or miR-520c gave rise to bone, pleural, and lung metastases in vivo upon injection into immunodeficient mice. Using in silico analysis, previous data on metastatic-associated genes, and miRNA target site analysis in luciferase reporter constructs, Ma et al demonstrated that the hyaluronan receptor, CD44, was a likely target of miR-373 and miR-520c.[68] As noted by the authors, CD44 expression is correlated with overall survival in breast cancer patients and inhibits metastasis in prostate and colon cancers. Huang et al.[68] observed that CD44-expressing MCF-7 cells promoted metastasis in vivo. Importantly, these investigators also demonstrated that removal of the 3′ UTR of CD44 mRNA antagonized the ability of miR-373 or miR-520c to promote migration of MCF-7 cells. In the context of human breast cancer, miR-373 expression was higher in metastatic lesions in 10 of 11 paired samples of primary and lymph node metastatic specimens and was elevated in general in metastatic versus nonmetastatic breast cancers. CD44 expression was lower in metastatic breast cancers, and CD44 levels were inversely correlated with miR-373 levels.

As discussed previously, miR-21 was reported to promote metastasis in colon cancer cells[58] and to down-regulate the tumor suppressors TPM1[50] and PDCD4.[51] Zhu et al[69] recently extended their work on miR-21 in breast cancer to show that knock-down of miR-21 in breast cancer MDA-MB-231 cells did not affect proliferation but significantly reduced cell invasiveness in vitro and lung metastases in vivo. This group also demonstrated that two tumor suppressors, PDCD4 and maspin, were direct targets of miR-21 (Table 1).

In contrast with studies in metastasis-promoting miRNAs, Tavazoie et al[35] recently identified miRNAs that inhibit breast cancer metastasis. This study examined miRNAs that have diminished expression in cell lines developed from bone and lung metastatic foci, relative to parental breast cancer MDA-MB-231 cells after injection of cells into mice. In this paradigm, the researchers were looking for miRNAs whose expression was not compatible with metastasis (i.e., their expression was selected against in the process of forming metastatic foci). Eight miRNAs were found to be significantly diminished, and the relative levels of two of these, miR-335 and miR-126, were highly correlated with metastasis-free survival among 20 primary breast tumors. Because miRNAs cause destabilization and degradation of some of their target mRNAs, the mRNA targets of miR-335 were identified by Tavazoie et al.[35] Restoration of miR-335 expression followed by mRNA profiling in lung metastasis–derived MDA-MB-231 cells (LM2 cells) revealed 756 genes whose levels were decreased by forced expression of miR-335. This group also identified 116 genes whose expression was increased in bone metastases–derived or lung metastases–derived cells compared with parental MDA-MB-231 cells. Comparison of these two sets revealed an overlap of six genes whose expression was increased in metastatic cells and decreased by forced expression of miR-335. Using bioinformatics and 3′ UTR–luciferase reporter assays, this set was reduced to four genes: (1) the transcription factor, SOX4; (2) the receptor-type tyrosine protein phosphatase, PTPRN2; (3) c-Mer tyrosine kinase, MERTK; and (4) the extracellular matrix component, tenascin C (TNC). In this study, SOX4 and tenascin C were singled out for further study.[35] SOX4 knockdown in the lung metastasis–derived LM2 cells induced a morphologic change in cells that was similar to forced expression of miR-335 and reduced migratory activity of the cells in vitro. TNC or SOX4 knockdown decreased the invasive activity of LM2 cells in vitro and completely blocked metastasis in a lung colonization assay.

Another important protein involved in the maintenance of epithelial polarity and a nonmigratory/noninvasive phenotype is E-cadherin.[70] Hurteau and colleagues demonstrated that the miRNA miR-200c targets the transcription factor TCF8, which in turn inhibits E-cadherin expression.[71] In the nonmetastatic, ERα-positive MCF-7 breast cancer cells, miR-200c and E-cadherin are endogenously expressed, whereas TCF8 expression is absent. Conversely, the metastatic, ERα-negative MDA-MB-231 cells express TCF8 but not miR-200c or E-cadherin. Forced expression of miR-200c in the latter cell line led to attenuated TCF-8 expression and restored E-cadherin expression and a more adherent, less transformed phenotype.

CONCLUSIONS

As discussed in the preceding reviews in this issue and in many reviews on miRNA function,[4,72] selective miRNAs can exert widespread and robust regulation on the expression of proteins in cells. As such, they hold much potential as targets of therapy directed at the correction of miRNA misexpression, as miRNAs represent a "one hit, multiple targets" object of therapeutics design.[73] Indeed, nucleic acid technology has advanced to a point where relatively stable nucleic acid–based molecules can be delivered into tumors to effect a change in their behavior in animals.[50,74] Thus, miRNA-targeted therapies may prove to be highly specific, efficacious, and cost-effective in the treatment of subtypes of breast cancer.[73]

ACKNOWLEDGMENTS

This research was funded by NIH grant R21-DK073456. We wish to acknowledge the Carole and Ray Neag Comprehensive Cancer Center at UConn Health Center for additional support.

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