MicroRNAs and Breast Cancer

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Abstract: MicroRNAs are a class of small non-coding RNAs that are involved in the negative regulation of gene expression primarily through binding to the 3'untranslated region of their target mRNA. MicroRNAs have multiple mRNA targets, each of which can be regulated by multiple microRNAs making this a complex regulatory network. The identification of their importance in different types of cancer and validation of their role in many processes related to cancer progression led to their description as a novel class of oncogenes (or 'oncomirs') and tumor suppressors. As a result, a massive influx of related research articles has emerged. Understanding the role of microRNAs in normal breast and cancer development and progression is critical to fully comprehend this disease. Many insights have been gained to date and this review highlights those with well established roles in breast cancer, as well as providing a comprehensive depiction of the most recent research articles in the field of microRNAs and breast cancer.

MICRORNAs: AN INTRODUCTION

MicroRNAs (miRNAs) are endogenous 19-25 nucleotide RNAs that have recently emerged as a novel class of small, evolutionarily conserved gene regulatory molecules involved in many critical developmental and cellular functions [1]. miRNAs base-pair with target mRNA sequences primarily in their 3' untranslated region (3'UTR). Through specific base pairing, miRNAs induce mRNA degradation, translational repression, or both depending upon the complementarity of the miRNA to its mRNA target. Each miRNA can target numerous (100-1000) mRNAs, often in combination with other miRNAs, therefore controlling complex regulatory networks. It is estimated that there are ~900 miRNAs in mammalian cells, and that at least 30% of all genes are regulated by miRNAs [2, 3]. Over 10,000 identified mature miRNAs exist in species ranging from plants to humans, suggesting that miRNAs are ancient players in gene regulation [4]. Their existence and conservation throughout species supports the concept that they perform critical functions in gene regulation [4]. Indeed, the conserved evolution of both miRNAs and transcription factors highlights their importance in and the complexity of gene regulation [5].

Although, the primary role for miRNAs is through base pairing in the 3'UTR of genes as described above, there is compelling evidence that miRNAs may also bind to regions in the coding sequence of genes (for a review [6]). In addition, there is evidence to suggest that miRNAs may lead to the activation of certain genes through binding to sequences in the 5'UTR of genes [7]. Cleary, the complexity of miRNA-mediated regulation is still not yet fully realized and we should anticipate many advances in this field in the not too distant future.

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What makes miRNAs particularly important is their involvement in most, if not all, fundamental biological processes [8-10]. Mounting evidence indicates that miRNAs may also play a significant role in cellular transformation and carcinogenesis acting either as oncogenes or tumor suppressors [11-16]. Furthermore, specific miRNA signatures have been identified for both solid cancers and hematologic malignancies [17-20]. Intriguingly, mounting evidence suggest that the power of miRNAs lies in the ability to distinguish specific cancer subtypes based on their miRNA profile, including, and of direct relevance to the studies described herein, breast cancer [17, 21]. Nonetheless, the identification and validation of specific targets has been limited.

MICRORNA BIOGENESIS

There have been reports of dysregulation of the biogenesis machinery in breast cancer. Transcriptional regulation of miRNAs is still not well understood; however, it is known that miRNAs can reside within genes (intronic) or between genes (intergenic) and studies have shown that they can be transcribed from both host and independent promoters [22]. The biogenesis of miRNAs begins with transcription in the nucleus by RNA polymerase II or III into primary miRNA (pri-miRNA) transcripts [23]. The pri-miRNAs are then cleaved by the microprocessor complex Drosha-DGCR8 (Pasha) in the nucleus to a ~70 nucleotide precursor or pre-miRNA. This precursor hairpin is then exported into the cytoplasm by Exportin 5 in complex with Ran-GTP [24]. In the cytoplasm the RNase III Dicer cleaves off the loop of the pre-miRNA and generates an ~22-nucleotide miRNA duplex. Following Dicer-mediated cleavage, a miRNP (miRNA-containing ribonucleoprotein particle) complex containing Argonaute protein 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 [25]. The other strand is degraded. This simple, linear view of miRNA biogenesis is sufficient for this review, but as with all processes the complexity involved is

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vast and is beyond the scope of this review and as described by Diederichs group, there are "Many roads to maturity" [26].

A recent study examined the biological and prognostic value of Dicer expression at both the RNA and protein level in breast cancer cell lines, breast cancer progression cellular models, and in two well-characterized sets of breast carcinoma samples obtained from patients with long-term follow-up using tissue microarrays and quantitative reverse transcription-PCR [27]. In summary, the study found that Dicer protein expression is significantly associated with hormone receptor status and cancer subtype in breast tumors (ER p=0.008; PR p=0.019; cancer subtype p=0.023, luminal A p=0.0174). In addition, they showed that Dicer mRNA expression appeared to have an independent prognostic impact in metastatic disease (hazard ratio=3.36, p=0.0032), suggesting that assessment of Dicer expression may facilitate prediction of distant metastases for patients suffering from breast cancer. Although studies on Dicer manipulation in breast cancer are limited, one study has shown that knockdown of Dicer by siRNA led to a significant G1 arrest and increased sensitivity to the DNA damaging agent, cisplatin, in MCF7 breast cancer cells, suggesting that over-expression of Dicer may be pro-tumorigenic [28].

MICRORNAs IN BREAST CANCER

The link between miRNAs and cancer attracted many researchers to this new and exciting field of research. The outcome resulted in a plethora of studies linking miRNA expression profiles with specific types of cancer, tumor subtypes, and even metastatic signatures from expression profiling and microarrays. In addition, functional studies have identified specific miRNAs as tumor suppressors and oncogenes and have linked their expression in breast cancer with the translational regulation of direct mRNA targets. The table below lists those miRNAs identified as playing a role in breast cancer (Table **1**); however, this review will highlight some of the more recent advances in miRNAs and breast cancer.

miR-21

Upstream Regulation

Since its initial discovery, miR-21 has become one of the most studied miRNAs in various fields of biology (for a detailed review see [51]). More recently, expression of miR-21 has been linked with the hyaluronan (HA)-induced interaction with CD44 and protein kinase C (PKC) epsilon in breast tumor cells [52]. HA binding to CD44 promotes PKC epsilon activation, which in turn increases the phosphorylation of the stem cell marker Nanog resulting in translocation from the cytosol to the nucleus and association with Drosha and p68 (RNA helicase). This process leads to the production of miR-21 and the subsequent decrease in PDCD4, a tumor suppressor protein and direct target of miR-21. The authors show that these events contribute to the up-regulation of IAPs (inhibitors of apoptosis proteins) and MDR1 (multidrug resistance protein), resulting in anti-apoptosis and chemotherapy resistance.

Estradiol (E2) was shown to inhibit miR-21 expression in MCF7 breast cancer cells [53]. They also showed that the E2-mediated decrease correlated with increased protein expression of endogenous miR-21 targets PDCD4, PTEN and Bcl-2. Conversely, a later study showed that miR-21 was induced in response to E2 along with 21 other miRNAs. Notably, 7 other miRNAs were reduced in the E2 treated MCF7 breast cancer cells [54]. The experimental differences (time in serum-free, charcoal stripped media and concentration and time of E2 treatment) may account for these effects. A recent study showed miR-21 to be correlated with HER2/neu up-regulation and functionally involved in HER2/neu-induced cell invasion via the MAPK (ERK1/2) pathway

Table 1. MicroRNAs and their Target Genes in Breast Cancer

MicroRNA	Expression and Role	Target	References
miR-21	Over-expressed, oncogenic	BCL2, TPM1, PDCD4, PTEN	[29-32]
miR-10b	Down-regulated, metastatic potential	HOXD10	[33]
miR-27a	Oncogenic (breast cancer cells)	ZBTB10	[34]
miR-206	Over-expressed	ER alpha	[35, 36]
miR-17-5p	Tumor suppressor (breast cell lines)	AIB1	[37]
miR-125a & b	Down-regulated, tumor suppressor	ERBB2, ERBB3	[38, 39]
miR-200 family	Down-regulated, metastasis suppressor	ZEB1, ZEB2	[40, 41]
miR-205	Down-regulated, tumor suppressor	ERBB3, VEGF-A, ZEB1, ZEB2	[40-43]
miR-146a & b	Metastasis suppressor	EGFR	[44]
miR-204	Over-expressed	PDEF	[45]
miR-510	Over-expressed	PDEF	[45]
miR-193b	Tumor suppressor (breast cell lines, xenograft)	uPA, ER alpha	[46, 47]
miR-145	Down-regulated	RTKN, ER alpha	[48, 49]
miR-155	Oncogenic	RhoA	[50]

upon stimulation of HER2/neu signaling in breast cancer cells [55]. Conversely, miR-21 has been shown to be repressed by BMP-6 (bone morphogenetic protein -6) through the E2-box and AP-1-binding sites within the promoter of miR-21 [56]. This study further showed using site-directed mutation and ChIP that miR-21 was activated by deltaEF1 by binding to the E2 box on the miR-21 promoter and furthermore that TPA triggered miR-21 activity through the AP-1 binding site.

Downstream Regulation

Direct targets of miR-21 were identified using a quantitative proteomic approach in MCF7 breast cancer cells [57]. This group observed an increase in the abundance of 58 proteins when miR-21 was inhibited. Validation of 12 of these candidate targets in luciferase assays showed that 6 of them were likely direct targets of miR-21, one of which included the known miR-21 target gene PCDC4. Of the 5 potentially novel targets identified in this screen, only 2/5 contained the 'required' 7mer seed, therefore are unlikely to be predicted by the bioinformatic databases available for target identification. This study also highlighted the importance of proteomic approaches of miRNA target identification as many of the targets identified in this study were not affected at the mRNA level.

Clinical Significance

Three studies recently examined the potential clinical significance of miR-21 expression in breast cancer. One study showed that miR-21 expression was associated with lymph node positivity (p=0.01), higher proliferation index (Ki67 > 10%; p=0.03) and advanced TNM clinical stage (p=0.021) using matched non-tumor and tumor tissues of 40 invasive ductal carcinomas of the breast. This study also reported a negative correlation with PTEN expression (p=0.013), a known downstream target of miR-21, in the same tissues [58]. The second study showed that high miR-21 expression was associated with features of aggressive disease, including high tumor grade, negative hormone receptor status, and ductal carcinoma, as well as a positive correlation with TGF-beta1 [59]. Although no association was found between patient survival and miR-21 expression among all patients, high miR-21 was associated with poor disease-free survival in early stage patients (HR = 2.08, 95% CI: 1.08-4.00) despite no value for prognosis. The third study performed global expression profiling of miRNAs in primary breast cancer (BC) and normal adjacent tumor tissues (NATs) and its potential relevance to clinicpathological characteristics and patient survival [31]. Among the 113 BC cases, this study found high level expression of miR-21 was significantly correlated with advanced clinical stage (p=0.006, Fisher's exact text), lymph node metastasis (p= 0.007, Fisher's exact text), and shortened survival of the patients (HR=5.476, p< 0.001). In addition, multivariate Cox regression analysis revealed this prognostic impact (HR=4.133, p=0.001) to be independent of disease stage (HR=2.226, p=0.013) and histological grade (HR=3.681, p=0.033).

miR-193b

Of interest, miR-193b was initially identified and further validated as a negative regulator of the estrogen receptor-alpha (ER alpha), in a high-throughput screen [46]. Recently identified miR-193b was shown to be differentially expressed between the MDA-MB-231 breast cancer cell line and its highly metastatic variant, the highly metastatic variant showing reduced expression of miR-193b [47]. Further analysis revealed that miR-193b repressed uPA protein expression and inhibited cell invasion through a binding site present within its 3'UTR. Furthermore, this study showed that miR-193b significantly inhibited the growth and dissemination of xenograft tumors in an immunodeficient mouse model and that miR-193b was a negative regulator of uPA in primary breast tumors.

miR-206

miR-206 was initially identified as a negative regulator of ER alpha mRNA and protein expression in breast cancer cell lines through two specific target sites within its 3'UTR and that miR-206 and ER alpha repress each other's expression in MCF-7 cells in a double-negative feedback loop [35]. Another group further showed that miR-206 expression is decreased in ER alpha-positive human breast cancer tissues and that its expression is inversely correlated with ER alpha, but not ER beta mRNA expression [36]. More recently, a study identified the co-activator proteins steroid receptor co-activator (SRC)-1 and SRC-3, and the transcription factor GATA-3, all of which contribute to estrogenic signaling and a Luminal-A phenotype as novel downstream targets of miR-206 [60]. Significantly, they showed that over-expression of miR-206 repressed estrogen-mediated responses in MCF-7 cells, even in the presence of ER alpha encoded by an mRNA lacking a 3'UTR, suggesting miR-206 affects estrogen signaling by targeting mRNAs encoding ER alpha-associated co-regulatory proteins.

miR-125b

Similar to the identification of other miRNAs, miR-125b was initially identified in a miRNA profiling study to define miRNAs that were aberrantly expressed between normal tissue and breast cancer [38]. In this study, miR-125b levels were found to be down-regulated in breast cancer when compared to normal, however no correlation was found with invasive breast cancer pathological features including estrogen receptor (ER), progesterone receptor (PR) and p53 status, tumor stage (pT), positive lymph nodes (pN) and low/high proliferation index (PI). Since then, multiple studies have reported down-regulation of miR-125b in breast cancer, particularly in ER positive tumors and suppression of ERBB2 and ERBB3 expression and function [39, 61-63]. Human vitamin D3 hydroxylase (CYP24), a candidate oncogene [64], was recently identified as a direct target of miR-125b [65]. Modulation of miR-125b levels resulted in altered CYP24 protein levels with no change in mRNA levels. Significantly, immunohistochemical analysis showed an inverse correlation between CYP24 protein levels and miR-125b levels in breast cancer/normal tissues, suggesting post-transcriptional regulation of CYP24 by miR-125b as a possible mechanism for high CYP24 protein expression in cancer tissues. More recently, a study identified a single nucleotide polymorphism (SNP) within a miR-125b binding site in the 3'UTR of the bone morphogenic receptor type 1B (BMPR1B) encoding a transmembrane serine/threonine

kinase [66]. They show that miR-125b negatively regulates BMPR1B and that C/T allelic variation within the target site disrupts this regulation. The presence of the T allele leads to loss of miR-125b regulation, increased BMPR1B expression, and ultimately elevated risk of breast cancer disease. This study is the first to suggest that genomic variation within miRNA target sites may be important sources for genetic differences in cancer risk.

miR-205

The aberrant expression of miR-205 has been demonstrated for multiple cancers [67-74]. Focusing specifically on breast, miR-205 has been shown to be a tumor suppressor; significantly, under-expressed in breast tumor compared to matched normal breast tissue [42, 43]. Functionally, ectopic expression of miR-205 inhibits cell proliferation, invasion and anchorage-independent growth in breast cancer cell lines through direct targeting of ERBB3 and VEGF-A [43]. Furthermore, together with members of the miR-200 family, miR-205 has been shown to regulate epithelial-mesenchymal transition (EMT) by targeting the E-cadherin transcriptional repressors ZEB1 and ZEB2, suggesting a potential role for miR-205 in cancer metastasis [40, 41]. The expression pattern of miR-205 was identified to be restricted to the myoepithelial/basal cell compartment of normal mammary ducts and lobules, whereas the expression was greatly reduced or eliminated in matching tumor specimens [75].

miR-204 and -510

Two miRNAs, miR-204 and miR-510, were recently identified as potential 'oncomirs' in breast cancer [45]. This study showed that the levels of miR-204 and miR-510 are elevated in human breast tumor samples when compared to matched non-tumor. In addition, this study shows that an increase in migration, invasion and colony formation occurred when miR-204 or miR-510 were over-expressed in the non-invasive breast cancer cell line MCF7. The ETS transcription factor and putative tumor suppressor, PDEF (prostate-derived ETS factor) was identified as a direct target for miR-204 and miR-510 with distinct and functional binding sites for each present in the 3'UTR.

Other miRNAs of Interest

Other recent studies of interest include the miRNA link made between breast cancer stem cells (BCSC) and normal stem cells. Specifically, 37 miRNAs were found that were differentially expressed between BCSCs and non-tumorigenic cancer cells. miR-200c modulated the expression of BMI1, a known regulator of stem cell self-renewal, and inhibited the clonal expansion of breast cancer cells *in vitro* [76]. Furthermore, this study showed that miR-200c suppressed the ability of normal mammary stem cells to form mammary ducts and tumor formation driven by BCSCs *in vivo*.

The breast cancer metastasis suppressor 1 (BRMS1) protein is able to suppress metastasis without affecting tumorigenesis. BRMS1 was shown to exert some of its anti-metastatic effects by regulating miRNA expression [77]. Specifically, the down-regulation of metastasis-promoting miRNAs -10b, -373 and -520c, and the up-regulation of metastasis suppressing miRNAs -146a, -146b and -335. In a separate study, miR-31 was identified as a metastasis suppressor [78]. In this study, inhibition of miR-31 allowed normally non-aggressiveness breast cancer cells to metastasize *in vivo*. They demonstrated that miR-31 can regulate multiple steps in the metastatic cascade independent of primary tumor development.

MICRORNAS IN NORMAL BREAST DEVELOPMENT

It is widely accepted that understanding the regulation of developmental processes results in a better understanding of how these pathways may become dysregulated and ultimately result in cancer. However, the role of miRNAs in normal breast development has not been well studied and much work is needed to understand their role in this highly complex process. Studies to date have shown that there is a human 'breast'-specific signature composed of 23 miRNA based on microarray data [79]. However, only 161 human and 84 mouse miRNA were present on this array and therefore a true representation of the miRNome remains to be established. In addition, RNA samples analyzed were from 18 adult and 2 fetal normal human tissues and therefore were not adequate to assess miRNA expression patterns during the complex and distinct stages of normal mammary development. More recently, a preliminary study was performed to analyze the expression pattern of 22 miRNAs in mouse mammary gland from various development stages [80]. Taken together with the fact that of these 22 miRNAs, over half (13/22) were not detected in the mammary gland, this is not a comprehensive enough study to assess the role of miRNAs in normal mammary gland development. Subsequent analyses from this group has performed a more complete analysis of miRNA expression in normal mammary gland development using a small RNA cloning method [81]. Using this technique, they were able to identify 3 known and 33 new miRNAs, some of which were differentially expressed during different stages of mammary gland development. Indeed, another study was performed using microarray and qRT-PCR to analyze the miRNA expression changes along the murine mammary cycle during pregnancy, particularly on transition from pregnancy to lactation [82]. This study illustrates that a miRNA expression pattern exists for each developmental stage of the mammary gland. Down-regulation of some miRNAs, such as miR-138 and miR-431, are observed in virgin compared to involution, whereas other miRNAs, such as miR-133 and miR-133a-133b, are up-regulated during pregnancy and lactation. These initial studies highlight the importance of miRNAs in normal developmental processes in the mammary gland and that future studies identifying direct targets and functional consequences of these differentially expressed miRNAs are essential to fully understand the normal developmental processes in the breast.

MICRORNA FUTURE DEVELOPMENTS

Diagnostic/Prognostic/Therapeutic

Specific miRNA signatures have been identified for both solid cancers and hematologic malignancies, and mounting

evidence suggest that the power of miRNAs lies in their ability to distinguish specific cancer subtypes based on their miRNA profile, including, and of direct relevance to this review, breast cancer. Patient stratification (i.e., selecting patients likely to respond to treatment and thus significantly improve response rates and potential patient survival) is an achievable objective if there is adequate knowledge of drug targets and their biology. Increasing evidence suggests that miRNAs regulate many of the biological processes associated with human disease, including cancer.

One of the major challenges in ASO development has been the instability of ASOs in serum. Modified synthetic antisense miRNA oligoribonucleotides (AMO) are useful tools in specifically inhibiting individual miRNAs, and are a proven resource in helping to unravel the function of miRNAs and their targets. Similar to antisense-based oligonucleotides (ASO), AMOs may contribute to the prioritization of pharmaceutical targets and have the potential to eventually progress into a new class of therapeutic agents. Another more recent advance in antisense technology is the emergence of peptide nucleic acids (PNAs) [83]. PNAs are artificial oligonucleotides constructed on a peptide-like backbone, that have a stronger affinity and greater specificity to DNA or RNA than natural nucleic acids and are resistant to nucleases, which is an essential characteristic for a miRNA inhibitor that will be exposed to serum and other cellular nucleases. PNA-based ASOs were also shown to be more effective miRNA inhibitors than other DNA-based ASOs, do not show non-specific cytotoxicity at concentrations up to 1000 nM and were shown to persist for 9 days *in vitro* [83]. Although PNA-ASO technology is moving in the right direction, these ASOs remain to be tested in an *in vivo* environment. The challenges for any *in vivo* inhibitors will be to control off-target effects, to design approaches for achieving a long duration of effectiveness, and to carefully titrate the expression level of a given miRNA so that it affects a selective, disease related endpoint.

ABBREVIATIONS

miRNA	=	MicroRNA
3'UTR	=	3' Untranslated region
pri-miRNA	=	Primary miRNA
miRNP	=	miRNA-containing ribonucleoprotein particle
ER	=	Estrogen receptor
PR	=	Progesterone receptor
PKC	=	Protein kinase C
E2	=	Estradiol
HA	=	Hyaluronan
IAPs	=	Inhibitors of apoptosis proteins
MDR1	=	Multidrug resistance protein
BC	=	Breast cancer
NATs	=	Normal adjacent tumor tissues
pT	=	Tumor stage
pN	=	Positive lymph nodes
PI	=	Proliferation index
CYP24	=	Human vitamin D3 hydroxylase
SNP	=	Single nucleotide polymorphism
BMPR1B	=	Bone morphogenic receptor type 1B
EMT	=	Epithelial-mesenchymal transition
PDEF	=	Prostate-derived ETS factor
BCSC	=	Breast cancer stem cells
BRMS1	=	Breast cancer metastasis suppressor 1
AMO	=	Antisense miRNA oligoribonucleotides
ASO	=	Antisense-based oligonucleotides

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