miRNAs in breast cancer tumorigenesis (Review)

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Abstract. miRNAs are small, endogenous, non-coding RNAs that negatively regulate protein-coding mRNAs at the post-transcriptional level. It is estimated that in humans thousands of miRNAs are expressed and more than 700 miRNAs have been described to date. About 50% of annotated human miRNAs are detected in regions of fragile sites, which are associated with cancer. The available evidence has shown that miRNAs widely participate in the development or progression of many types of cancers, including breast cancer. The role of miRNAs in breast cancer has been widely investigated; here, we will focus on what is known about the working mechanism of miRNAs in different stages of breast cancer development.

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1. Introduction

miRNA was initially discovered as a small temporal RNA (stRNA) in C.elegans in 1993 (1). However, not much attention was paid to this finding until seven years later when Let-7 (the second miRNA) was identified (2). In the following years, researchers became aware that miRNAs were a large family of small non-coding RNAs that exist in species ranging from plants to humans (3-8). Correspondingly, the functions of miRNAs were found to not be limited to temporal regulation, but were shown to be implicated in various biological processes, including cell cycle (9,10), proliferation (11), apoptosis (12,13) and development (6). In 2002, Calin and colleagues (14) reported the first direct evidence of miRNA playing a role in human cancer; they found that miR-15 and miR-16 contribute to chronic lymphocytic leukemia. Subsequently, more examples of miRNA correlated with human cancers were noted. Iorio and colleagues (15) first demonstrated miRNA dysregulation in human breast cancer by miRNA microarray; they found that miR-10b, miR-125b, and miR-145 were down-regulated, while miR-21 and miR-155 were up-regulated, suggesting that these miRNAs may act as potential tumor suppressor genes or oncogenes, respectively. Following this finding, more functional studies had identified specific miRNAs as pivotal regulators in different stages of breast cancer development (initiation, progression and metastasis). In this review we will summarize the current understanding regarding the functions of miRNAs in breast cancer tumorigenesis.

Finally, based on these experimentally validated specific breast cancer-associated miRNAs and their gene targets, we summarize stage-specific miRNA functions of breast-derived cells and tissues in different phases (Table I). This approach reveals that some miRNAs, such as miR-21, play a key role in all phases of breast cancer tumorigenesis. Other miRNAs, such as miR-30, miR-17-5p, miR-9, are phase-specific. It suggests that regulation of miRNAs themselves at specific stages may be crucial for breast cancer tumorigenesis.

2. microRNAs as regulators in breast cancer initiation

Currently, it is universally acknowledged that cancers may arise from cancer stem cells, also termed as tumor-initiating cells (T-IC), which are the primary cellular components within a tumor that drives disease progression and are characterized by their stem-like ability to self-renew (16). Evidence of breast cancer-initiating cells (BT-IC) was reported by Al-Hajj and Clarke (17). Such cells may be responsible for breast cancer initiation. However, how the self-renewal of BT-ICs is regulated remains obscure. A previous study showed that the regulation of the self-renewal of breast cancer stem cells is associated with the Hedgehog pathway. Bmi-1 is a downstream target of Hedgehog pathway. Bmi-1 has been shown to be required for self-renewal (18,19). These findings clearly indicate that the Hedgehog pathway activates breast cancer stem cell self-renewal by Bmi-1. A recent study has implicated several miRNAs in the regulation of BT-IC self-renewal. These miRNAs include miR-200c, Let-7, miR-30, but presently, little is known about the mechanism by which it functions to regulate BT-IC self-renewal. What is clear is...
Table I. microRNAs and their targets in different phases.

| Phase      | microRNA | Expression and role                      | Target                      | Refs. |
|------------|----------|------------------------------------------|-----------------------------|-------|
| Initiation | miR-200c | Down-regulated, BT-IC self-renewal suppressor | Bmi-1                       | (20)  |
|            | Let-7    | Down-regulated, BT-IC self-renewal suppressor | Ras                         | (21)  |
|            | miR-30   | Down-regulated, BT-IC self-renewal suppressor | Ubc9, integrin β3           | (22,23) |
| Progression| miR-21   | Up-regulated, anti-apoptotic factor        | Unknown                     | (33)  |
|            | miR-145  | Down-regulated, inducing apoptosis         | RTKN                        | (35)  |
|            | miR-155  | Up-regulated, anti-apoptotic factor        | FOXO3a                      | (36)  |
|            | miR-34a  | Down-regulated, inducing apoptosis         | Bcl-2                       | (39)  |
|            | miR-17/20| Down-regulated, proliferation suppressor   | Cyclin D1                   | (41,42) |
|            | miR-27a  | Up-regulated, inducing proliferation       | Myt-1, ZBTB10               | (43)  |
|            | miR-17-5p| Down-regulated, proliferation suppressor   | AIBI                        | (44)  |
|            | miR-106b/93/25 | Up-regulated, inducing proliferation | pRb | (45)  |
|            | miR-206  | Up-regulated, inducing proliferation       | ERα                         | (47)  |
|            | miR-18a/b/221/222| Up-regulated, inducing proliferation | CD44 | (53)  |
|            | miR-21   | Up-regulated, inducing proliferation       | PTEN                        | (50)  |
| Metastasis | miR-205/200 | Down-regulated, EMT suppressor            | ZEB1, ZEB2                  | (59)  |
|            | miR-31   | Down-regulated, EMT suppressor             | RhoA                        | (60)  |
|            | miR-155  | Up-regulated, inducing EMT                 | E-cadherin                  | (61)  |
|            | miR-21   | Up-regulated, inducing EMT                 | TIMP1,TIMP3 PDCD4           | (66,67) |
|            | miR-373 /miR-520c | Up-regulated, inducing EMT | CD44 | (53)  |
|            | miR-335 /miR-126 | Up-regulated, inducing EMT | TNC | (77)  |
|            | miR-10b  | Up-regulated, inducing EMT                 | HOXD10                      | (80)  |
|            | miRNA-672 | Down-regulated, EMT suppressor            | PRDX6                       | (83)  |
|            | miR-126  | Down-regulated, angiogenesis suppressor    | VEGF                        | (87)  |
|            | miR-9    | Up-regulated, inducing EMT and angiogenesis| E-cadherin                  | (57)  |
|            | miR-20b  | Down-regulated, angiogenesis suppressor    | HIF-1α                      | (92)  |

BT-IC, breast cancer-initiating cells; EMT, epithelial-mesenchymal transition.

Figure 1. A simple model for regulation of breast cancer initiating cells (BT-IC) self-renewal in breast cancer. The Hedgehog (HH) pathway regulates the self-renewal of BT-IC via the downstream targets: Patched (PTCH), Smoothened (SMO), Suppressor of Fused (SUFU), CLI and Bmi-1. miR-200c is also associated with this pathway. miR-30 and Let-7 may modulate BT-IC self-renewal by the pathway shown here.

that miR-200c strongly suppressed the ability for self-renewal of breast cancer stem cells (20). Studies also show that lack of Let-7 is required for self-renewal in breast cancer stem cells; moreover, Ras is determined as the direct target of Let-7 and its silencing contributes to loss of BT-IC self-renewal (21). More recently, it is demonstrated that up-regulated expression of miR-30 in breast cancer-initiating cells inhibits their self-renewal capacity by reducing the ubiquitin-conjugating enzyme 9 (Ubc9). Ubc9 has been shown to be specific for small ubiquitin-related modifier (SUMO) activation. SUMO may up-regulate Oct4 by stabilizing its structure (22,23). Oct4 overexpression induced by Ubc9 contributes to the self-renewal (24). Integrin β3 (ITGB3) is another direct target of miR-30, which contributes to apoptosis (22). A previous report has shown that unligated ITGB3 recruits caspase-8 to the cell membrane and activated caspase-8 mediates apoptosis in a death receptor-independent manner (25), while miR-30 induces apoptosis not in the death receptor-independent manner but through an unclear pathway. In addition, a recent study indicates that integrins play a role via directly regulating the ability of BT-ICs to self-renew during the initial steps of breast cancer tumorigenesis (26). These findings support two greatly simplified models of regulation of BT-IC self-renewal (Fig. 1). The identification of miRNAs functioning as regulators of BT-IC self-renewal partially expand our understanding of the regulation of breast cancer initiation.

Although the molecular mechanisms by which miRNAs play a crucial role in tumor progression and metastasis have
been studied in great detail over the last decades, the role of miRNAs in the early events of tumorigenesis has only recently been demonstrated. As tumor formation is a multi-step process, the initiating events may facilitate the development of effective targeted therapeutic strategies for cancer.

3. Roles of microRNAs in breast cancer progression

Cancer stem cells drive tumor progression and heterogeneity by proliferating and generating some differentiated cancer cells (27). These differentiated cancer cells will gain the ability to anti-apoptosis and full out of the control of the normal cell cycle during cancer progression. Here, we will highlight those miRNAs identified as regulators of anti-apoptosis and of the cell cycle in breast cancer progression.

Anti-apoptosis. In normal breast tissue, apoptosis plays a key role for performing the normal functions. The mechanism of apoptosis still needs to be fully investigated. There is evidence that mitochondria play an essential role in the apoptotic process (Fig. 2). Several pathways contribute to apoptosis, but the best characterized are the Akt/PKB pathway, RTKN/NF-κB survival pathway and the p53-mediated apoptosis pathway (28-30). Emerging research shows that miRNAs are involved in these pathways. Bcl-2 family proteins can be thought of as the central factors of the apoptotic pathway. The Bcl-2 family is comprised of many proteins, which can be classified into three functional groups. Group I members, including Bcl-2 and Bcl-xL, possess anti-apoptotic activity; group II members, including Bax and Bak, are characterized by pro-apoptotic activity; group III members, such as Bim and Bad, also possess pro-apoptotic activity (31). After the identification of the down-regulation of miR-15/16 promoting anti-apoptosis via up-regulating Bcl-2 expression in leukemias and lymphomas, more miRNAs promoting anti-apoptosis by directly or indirectly regulating Bcl-2 family proteins have been observed in many types of cancer, including breast cancer (32).

Studies by Si and colleagues (33) showed that treatment of MCF-7 breast cancer cells with anti-miR-21 causes cell apoptosis. Moreover, they detected a lower level of Bcl-2 and Bcl-xL, will keep cytochrome c in the mitochondria inhibiting the apoptotic pathway. The Ubc9/ITGB3 pathway activates apoptosis in a mitochondria-independent manner.

Figure 2. Regulation of apoptosis by several pathways. When pro-apoptotic members of the Bcl-2 family, such as Bax and Bak, are activated by some pathway, mitochondria release cytochrome c. Cytochrome c in turn associates with Apaf-1 and then caspase to trigger apoptosis. Anti-apoptotic members, such as Bcl-2 and Bcl-xL, will keep cytochrome c in the mitochondria inhibiting the apoptotic pathway. The Ubc9/ITGB3 pathway activates apoptosis in a mitochondria-independent manner.
Cell cycle dysregulation. Cancer cells are characterized by deregulated cell proliferation during cancer progression. Proliferation is controlled by cell cycle in normal tissues (40). Numerous regulatory pathways contribute to the cell cycle and their alterations are necessary for cancer cells to overcome the control of the normal cell cycle (Fig. 4). miRNAs may alter the cell cycle by controlling regulators of these regulatory pathways. In this section, the roles of miRNAs in breast cancer cell cycle regulation will be discussed.

The cyclin/CDK (cyclin dependent kinase) pathway is an important pathway in the regulation of the cell cycle. This pathway can be regulated by several miRNAs in breast cancer and in cell lines. For example, the miR-17-5p/miR-20a miRNA cluster is shown to attenuate cyclin D1 through directly combining with the 3'-UTR binding site in MCF-7 cell line, thereby inhibiting S-phase entry and halting cell proliferation. Correspondingly, the miR-17/20 cluster is down-regulated and promotes cell proliferation in breast cancer cells (41). Further studies reveal a novel regulatory mechanism in which cyclin D1 induces an miRNA signature including miR-17/20 through the binding of the miR-17/20 promoter region (42). In addition to the miR-17/20 cluster, miR-27a is also associated with the cyclin/CDK pathway. ZBTB10 and Myt-1 are identified as direct targets of miR-27a. ZBTB10 (a putative Sp repressor) can inhibit the proliferation of breast cancer cells by suppressing cyclin D1 indirectly and Myt-1 can block cell cycle progression at the G2/M phase through suppression of cyclin B (43). E2Fs are critical regulators of the cell cycle; they can activate the expression of the miRs-106b/93/25 cluster. E2F is a downstream target of pRb and miRs-106b/93/25 can silence pRb. Furthermore, miR-17-5p is down-regulated in breast cancer cell lines, which has been shown to limit the oncogene AIB1, which enhances the transcriptional activity of the estrogen receptor (ER) and E2F1, leading to proliferation suppression (44). Thus, a negative feedback loop is generated (Fig. 5) (45).

Another cell cycle regulatory pathway in breast cancer is the E2/ERα/Sp1 pathway (46). Upon activation of the receptor estrogen receptor α (ERα), the pathway ERα/Sp1 enhances proliferation via activating cyclin D1, which eventually leads to the G1/S-phase transition. The regulation between the ER and miRNAs has been extensively investigated.

miR-206 is up-regulated in ERα-negative breast tumors and cell lines and inhibits ERα translation by binding to the 3’UTR of ERα mRNA (47). In addition to miR-206, ERα mRNA is also a direct target of miR-18a, miR-18b, miR-193b, miR-302c and miR-221/222 in breast cancer cells. Similar to miR-206, miR-18a, miR-18b and miR-221/222 are also up-regulated in ERα-negative cell lines, suggesting an important role of these miRNAs in the development of ERα-negative breast cancers.
of prometastatic genes. RhoA is one of such genes, which may prevents metastasis at multiple steps by inhibiting the expression of miR-205 directly target ZEB1 and ZEB2, suggesting that RhoA contribute to the EMT in different cell lines, suggesting that RhoA regulates EMT in a multiphasic manner.

For invasion to take place, cyclic attachment to matrix components must be released. Metalloproteinases (MMP) play an important role in this event. MMP can degrade the ECM, which is the extracellular part of tissue and mediates cell attachment (62). The tissue inhibitor of metalloproteinases (TIMPs) inhibits the activity of MMP (63) and contains a consensus miR-21 binding site. Previous study reported that miR-21 directly targets TIMP3 in glioma cells and leads to increases of their migratory and invasive abilities (64,65). A recent study showed for the first time that miR-21 negatively regulates TIMP3 expression in breast cancer via the binding of the 3'UTR of TIMP3 mRNA and promotes breast cancer invasion in multiple cell lines in vitro (66). In addition, miR-21 also affects invasion and metastasis by directly suppressing expression of tropomyosin 1 (TPM1), PDCD4 and maspin (67). As an actin-binding protein, TPM1 is capable of stabilizing microfilaments and controlling cell motility (68). The actin microfilaments are components of the cytoskeleton, and mediate a variety of essential biological functions in all eukaryotic cells, including providing the driving force for cells (69). TPM1 mRNA expression has been shown to be reduced in the metastatic breast cancer MDA-MB-231 and MDA-MB-435 cell lines and in metastatic colon cancer SW620 cell line (70). These facts suggest that suppression of TPM1 expression by miR-21 is a general way for metastatic tumor cells to disrupt the ECM and contribute to metastasis. Similarly, PDCD4 expression is blocked by miR-21 in breast cancer and colon cancer, suggesting that this interplay may be a general carcinogenic pathway, rather than a tissue-specific mechanism (71,72). The mechanism by which PDCD4 regulates cell invasion remains unclear, however, present evidence supports that PDCD4 inhibits AP-1 by binding to the eukaryotic translation initiation factor 4A (eIF4A). Subsequently, AP-1 and other cis-acting elements together interact with the AP-1 site at the MMP promoter (73,74). Earlier evidence suggests that miR-373 and miR-520c can stimulate migration and invasion of MCF-7 and MDA-MB-435 cells, at least in part through direct suppression of CD44 (53), which functions as a cell surface receptor for several ECM components and mediates cell-cell or cell-substrate interactions through recognition of elements of the ECM (75,76). In addition, miR-335 and miR-126 are also reported to be associated with the ability of breast cancer cells to metastasize to the lung and bone by directly suppressing the ECM component tenascin c (TNC) (77). Taken together, these observations reveal that miRNA can induce cell migration and invasion by directly degrading the ECM or disrupting recognition between the cell and the ECM.

RhoC is an extensive researched prometastatic gene (78,79), which is reported a member of the Ras superfamily of...
small GTPases, playing a role in modulating assembly of actin-myosin contractile filaments and focal adhesion complex. Ma and colleagues (80) initially observed that miR-10b is highly expressed in metastatic breast cancer cells and they further found that the miR-10b is induced by Twist and it inhibits translation of the mRNA encoding HOXD10 (a homeobox transcription factor that promotes or maintains a differentiated phenotype in epithelial cells). RhoC increases with the decrease of HOXD10 leading to tumor cell invasion and metastasis (80,81). A later study indicated that RhoC is dispensable for tumor initiation but essential for metastasis (78). As a protein with both glutathione peroxidase and phospholipase A2 activities, peroxiredoxin PRDX 6 was previously described playing a crucial role in reactive oxygen species (ROS) resistance. Lehtonen et al. (82) first demonstrated that PRDX may be associated with human lung carcinoma. Chang and colleagues (83) found that PRDX is up-regulated in highly invasive and potentially metastatic MDA-MB-231 HM breast cancer cells compared with their parental cells. Furthermore, they demonstrated that overexpression of PRDX6 in breast cancer cells promoted their invasive and metastatic potential in vitro and in vivo (82,84). RhoC was up-regulated and TIMP-2 was down-regulated, in the cells with up-regulation of PRDX6 (83). When PRDX6 was knocked down by miRNA-672, RhoC was down-regulated and TIMP-2 was up-regulated (83). These findings indicate that miRNA-672 indirectly regulates breast cancer cell invasiveness and metastasis via down-regulating PRDX6 expression. However, how PRDX6 regulates RhoC and TIMP-2 as well as whether PRDX6 is an instigator of metastasis or merely a correlative product during progression of breast cancer are still beyond present understanding (85). As the available knowledge has shown that PRDX6 functions as an anti-oxidative protein to protect cells from damage by ROS, therefore it is reasonable to believe that ROS may play a key role in the regulation of RhoC and TIMP-2 by PRDX6. Further study into the mechanism of these relationships may add to the current state of knowledge of these signaling pathways, and will improve our understanding of metastasis-related interaction between miRNAs and cancer protein-coding genes.

To obtain sufficient nutrients and oxygen for metastasis of solid tumors, the formation of new blood vessels (angiogenesis) is necessary (86). It is now well established that tumor-induced angiogenesis is driven by the overexpression of angiogenic factors such as vascular endothelial growth factor (VEGF), which is the most potent inducer of angiogenesis. Of their wide range of biological actions, the role of miRNAs in tumor angiogenesis has received the greatest attention. Recent studies have shown that VEGF-A may be well-regulated by miR-126 in normal tissues and miR-126 was restrictively expressed in human breast cancer where the VEGF/P13K/AKT signaling pathway was vigorously activated. In addition, miR-126 directly targeted VEGF and its expression was decreased in human breast cancer, revealing that miR-126 plays a role in angiogenesis (87-90). Yet, another pathway regulating VEGF expression was presented by Ma et al. (57) who described that the up-regulation of VEGF-A mRNA by miR-9 depends on its ability to down-regulate E-cadherin expression and to activate β-catenin-mediated transcription. E-cadherin has been identified as the direct target of miR-9 and VEGF-A has been described as a transcriptional target gene of β-catenin. The data illustrates a novel mechanism by which miR-9 promotes angiogenesis through stimulation of VEGF-A expression in breast cancer. A recent study proposed that the VEGF expression in breast cancer cells is mediated by HIF-1 in a miR-20b-dependent manner. Hypoxia is one of the features within the tumor microenvironment. Hypoxia inducible factor1 (HIF-1) is a heterodimeric transcription factor consisting of HIF-1α and HIF-1β subunits (91,92). Under oxygenated conditions, HIF-1α is rapidly degraded, while in hypoxic conditions, this factor is stabilized and contributes to angiogenesis by directly activating the VEGF gene (93). Taking into account the above discussed evidence on the involvement of miRNAs in breast cancer-induced angiogenesis it would be of interest to address the functional relationship among these miRNAs. These regulators adjust the same target-VEGF by different pathways under specific conditions and trigger angiogenesis. One interesting question to be addressed is whether these miRNAs share the same specific expression pattern or not. Therefore, more novel miRNAs which participate in the process of VEGF-mediated angiogenesis in breast cancer should be identified to understand these expression patterns.

5. Conclusion

Breast cancer develops because of complex multistep processes. Generally speaking, there are three phases (initiation, progression and metastasis) in the complex multistep process. These phases are composed of a sequence of events, including self-renewal apoptosis, cell cycle and mobility. miRNAs are an evolutionarily conserved class of small, approximately 22-nucleotide non-coding RNAs that decrease gene expression post-transcriptionally in a sequence-specific manner, which participates in these events and some members extensively contribute to breast tumorigenesis. Over the past years, the utilizations of high-throughput technologies, such as microarray, a large number of ectopic miRNAs have been observed in breast cancer but the critical roles of most of these miRNAs remain largely unknown. Among these miRNAs, one miRNA can potentially regulate the expression of hundreds of genes, and on the other hand, a single transcript can be targeted by multiple miRNAs. However, knowledge of how an miRNA simultaneously down-regulates multiple proteins in the same pathway and an understanding of the miRNA target genes and their biologic functions is limited. There is no doubt that to further understand breast cancer pathogenesis, identifying the genome-wide targets of these miRNAs is essential. In addition, identifying the factors determining tissue- and cell-specific expression of miRNAs is also pivotal. As described above, miRNAs may act cooperatively through multiple target sites in one gene (94), or one miRNA may regulate a group of functionally related genes. Interestingly, unwanted cross-reaction does not appear in these two regulatory patterns. In addition, the mechanism does not seem to be specific of miRNA itself but the specific expression of miRNA. If only partially complementary sequences exist, targets will be repressed, regardless of the target gene specificities. In fact, miRNAs are expressed in specific cells and tissues at specific developmental stages and conditions. These facts clearly show that the system determining the specific expression of miRNAs plays a key role in regulating gene expression. In
recent years, numerous miRNAs and their targets have been confirmed in breast cancer and have been recognized as new therapeutic targets. However, these new therapeutic targets may not work, for the destruction of interplay between one miRNA and its target will be restored by another miRNA with similar function. Here, we postulate that the miRNA specificity determining system is composed of the effective targets, and miRNAs function as signal molecules together with other regulatory elements mediating breast cancer tumorigenesis in a stage-specific manner. Nevertheless, at the current stage little is known about these systems and various aspects of them need to be clarified in a future study. Taken together, the miRNA specificity determining system may serve as more effective potential target for breast cancer therapy in comparison with miRNA.

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