MicroRNAs: The Master Regulators of the Breast Cancer Tumor Microenvironment

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Abstract

Breast cancer is the most commonly diagnosed cancer globally and is among the leading causes of cancer deaths worldwide. Breast cancer mortality rates are increasing due to delays in diagnosis, prognosis, and treatment caused by the coronavirus disease 2019 (COVID-19) pandemic. Identification and validation of blood-based breast cancer biomarkers for early detection is a top priority worldwide. MicroRNAs (miRNAs) show the potential to serve as breast cancer biomarkers. miRNAs are small, endogenously produced RNAs that regulate growth and development. However, oncogenic miRNAs also play a major role in tumor

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growth and can alter the tumor microenvironment (TME) in favor of cancer metastasis. The TME represents a complex network of diverse cancerous and noncancerous cell types, secretory proteins, growth factors, and miRNAs. Complex interactions within the TME can promote cancer progression and metastasis via multiple mechanisms, including oxidative stress, hypoxia, angiogenesis, lymphangiogenesis, and cancer stem cell regulation. Here, we decipher the mechanisms of miRNA regulating the TME, intending to use that knowledge to identify miRNAs as therapeutic targets in breast cancer and use miRNAs as blood-based biomarkers.

Keywords

Breast cancer · MicroRNA (miRNA) · Tumor microenvironment (TME) · Oxidative stress · Reactive oxygen species (ROS) · Angiogenesis · Hypoxia · Cancer stem cells (CSCs) · Secretome · Biomarker

Introduction

Cancer is the collection of diseases where cells divide rapidly and uncontrollably, spreading to surrounding tissue, forming malignant tumors. Cancer incidence and mortality continue to rise in this era. The latest statistics estimate that 1 in 5 individuals will develop the disease in their lifetime, and 1 in 8 men and 1 in 11 women will die from it (Sung et al. 2021). In 2020, 19.3 million new cancer diagnoses led to nearly ten million deaths, making cancer one of the leading causes of death worldwide. It is anticipated that there will be 28.4 million new cancer cases in 2040, leading to a 47% increase from 2020. Additionally, cancer mortality is expected to increase due to delays in diagnosis, reduced healthcare access, and lack of treatment available during the coronavirus disease 2019 (COVID-19) pandemic.

Breast cancer is the most prevalent organ-specific cancer and accounts for 30–40% of total cancer diagnoses in women under the age of 40 in North America (American Cancer Society 2019). While breast cancer accounts for about 15% of all cancer-related deaths in women, early detection and treatment strategies have contributed to reducing disease-related mortality. Cancer detection has improved significantly with advancements in diagnostics and molecular tools; however, metastasis is currently responsible for up to 90% of cancer fatalities (Dillekås et al. 2019). Despite promising progress in breast cancer treatments with surgery, chemotherapy, radiation, and hormone therapy, problems such as post-treatment reactions, toxicities, and side effects associated with drug resistance are still crucial concerns for patients' quality of life. The success of clinical trials targeting various mutator phenotypes and responses to specific therapeutic targets is dependent on the tumor microenvironment (TME) and is a major challenge in the success of personalized therapy (Loeb 2011). Exploring the TME in clinical settings has had beneficial effects on other malignancies and tremendously improved patient treatment

regimens and outcomes. The TME is becoming a point of focus in breast cancer research and needs detailed investigation.

Alterations in nonprotein-coding transcripts, such as microRNAs (miRNAs), are emerging as molecular regulators of the TME (Majumder et al. 2015; Majumder et al. 2018a; Shin et al. 2019; Hunter et al. 2019; Liu et al. 2019; Gervin et al. 2020, Pan et al. 2020). Since miRNAs are major regulators of the TME and exploring the TME has a favorable clinical relevance in breast cancer, studying miRNA biology within the TME could improve breast cancer treatment strategies and reduce breast cancer mortality. miRNAs can be detected in patient blood samples, making miRNAs ideal candidates to be used as a molecular tool for noninvasive breast cancer detection (Hamam et al. 2017; Majumder et al. 2021; Filipów and Łaczmański 2019). In this chapter, we discuss the roles of miRNA within the TME in breast cancer.

Breast Cancer Overview

Breast cancer is defined by abnormal cell growth within the breast tissue. Although breast cancer can occur in both sexes, it is most common in females, accounting for 1 in 4 cancer cases and 1 in 6 cancer deaths (American Cancer Society 2019). In 2020, there were 2.26 million breast cancer diagnoses and 684,996 breast cancer deaths globally, making breast cancer the most prevalent organ-specific cancer in humans at 11.7% of total cancer cases (Sung et al. 2021).

The human breast is made up of three primary parts: tubes that carry milk to the nipple (ducts), glands that produce milk (lobules), and connective tissue (Fig. 1a). Breast cancer most commonly develops in epithelial cells of the ducts or lobules, forming breast carcinomas. The disease can be localized to the breast (in situ), or malignant cells can break through the walls of the ducts or lobules and metastasize (become invasive), allowing breast cancer to be categorized into different stages of severity. The tumor, lymph node, and metastasis (TNM) staging system is most commonly used in breast cancer to classify tumor severity between stages 0 and IV (Canadian Cancer Society 2021). The T category describes the presence and size of the original tumor, N explains whether cancer has spread to nearby lymph nodes, and M indicates distant metastases with pathological confirmation. As breast cancer stages increase, the severity of the disease and mortality rise, as reflected by the stagewise 5-year survival rates (Fig. 1b). Stage 0 and I have a 100% 5-year survival rate, followed by stage II, stage III, and stage IV, having 84.6%, 59.0%, and 26.5% 5-year survival rates, respectively (SEER Explorer 2021).

Breast Cancer Subtypes

Breast cancer is categorized into four major subtypes based on the presence or absence of growth factors and hormone receptors. The three main receptors found on breast tumor cells are the estrogen receptor (ER), the progesterone receptor (PR),

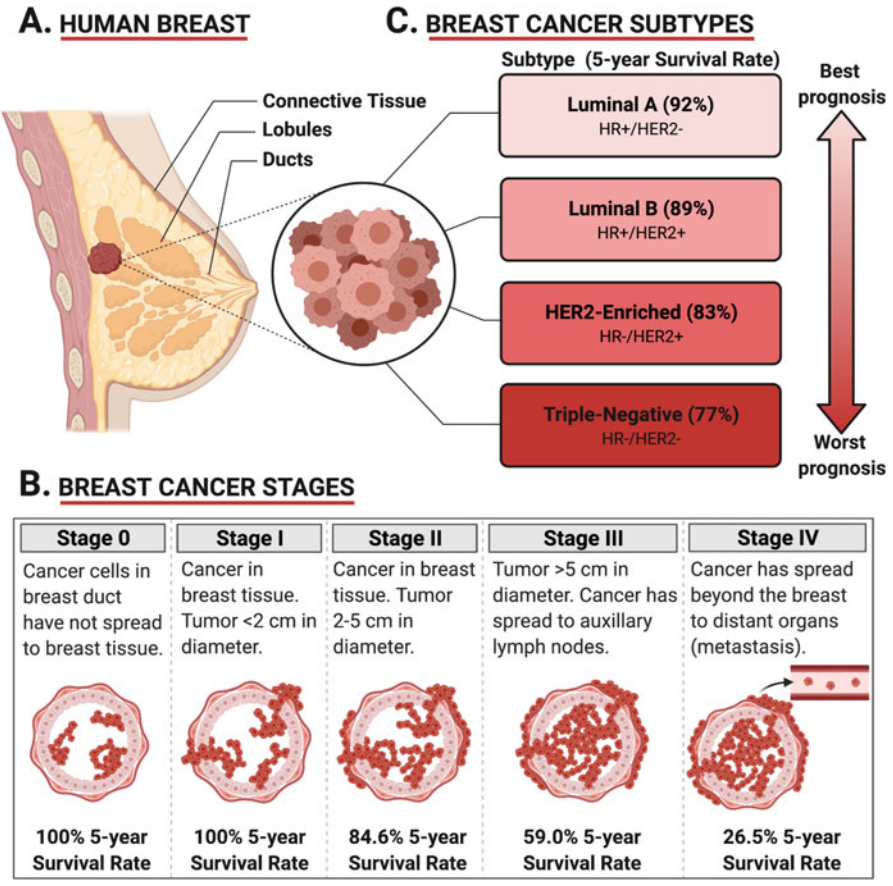

Fig. 1 Breast cancer summary. (**a**) Human breast anatomy. (**b**) Breast cancer staging and their corresponding 5-year survival rates. (**c**) Breast cancer subtypes and their associated 5-year survival rates

and the human epidermal growth factor receptor 2 (HER2). ER and PR statuses are often combined and classified as the hormone receptor (HR). The status is considered HR+ if patients test positive for the ER, the PR, or both receptors. Depending on the patients' receptor status, breast cancer can be classified into four subtypes, luminal A (HR+/HER2-), luminal B (HR+/HER2+), HER2-enriched (HR-/HER2+), and triple-negative (HR-/HER2-) (Fig. 1c). Luminal A is the most commonly diagnosed breast cancer subtype at 73% but has the highest 5-year survival rate of 92% (American Cancer Society 2019). Triple-negative breast cancer is diagnosed in 12% of patients but has the lowest 5-year survival rate of 77%. In comparison, luminal B and HER2-enriched breast cancer subtypes follow with 11% and 4% of diagnoses and 89% and 83% 5-year survival rates, respectively. Drug resistance in ER+ breast cancer remains relatively common, and interactions within the tumor microenvironment (TME) are a

driving factor (Shee et al. 2018). Thus, a greater understanding of the TME may lead to better forms of receptor-specific breast cancer detection and treatment regimens.

Current Breast Cancer Screening

Breast cancer is typically detected when a lump in the breast is noticed by self-examination or during a breast screening process. The disease usually has no symptoms when the tumor is small and in an early stage (stage 0 or I). If detected at these early stages, the survival rate of patients is 100%, making early breast cancer detection of great importance for patient survival (American Cancer Society 2019). Many countries offer mammogram screening programs; however, there is no global standard. The consensus recommendation for mammogram screening begins at age 50 and is suggested every 2 years after (Ebell et al. 2018). When cancer is suspected during preliminary screening, tissue is obtained from the tumor site with a needle or surgical biopsy for microscopic analysis. Both mammograms and biopsies are considered painful and invasive, and there are few proven interventions to reduce the pain and discomfort to patients. Furthermore, the rate of false-positive and false-negative mammography test results is high, adding to the limitations of mammographic screening (Le et al. 2016; Houssami and Hunter 2017). Moreover, breast cancer incidences are increasing in younger populations; thus, finding and validating a less invasive breast cancer diagnostic tool, like a blood-sensitive biomarker, is the number one priority in breast cancer research. There are many potential candidates from biomarkers in breast cancer including noncoding RNAs such as miRNAs. miRNAs are endogenously produced small RNA molecules that can be detected in blood and show potential as blood-based cancer biomarkers (Hamam et al. 2017).

miRNA Biogenesis

miRNAs are a class of small (19–25 nucleotides), evolutionary conserved, nonprotein-coding, single-stranded RNAs that mediate numerous biological processes by regulating gene expression at the posttranscriptional level (Hamam et al. 2017; Oliveto et al. 2017). miRNAs negatively regulate gene expression through selective binding to complementary target sequences in messenger RNAs (mRNA), thereby interfering with translational machinery and leading to decreased or inhibition of protein expression. Families of miRNAs are often found in groups within the genome, known as miRNA clusters, displaying similar levels of expression and function. Analysis of particular miRNAs, along with their respective miRNA clusters, is important for understanding the roles of specific miRNA families in normal physiology and mechanisms of disease.

Genes of miRNAs are transcribed in the nucleus by RNA polymerase III (POL III) to produce primary miRNA (pri-miRNA) transcripts (Oliveto et al. 2017) (Fig. 2). Pri-miRNAs are further processed by the ribonuclease III enzyme Drosha and dsRNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8)

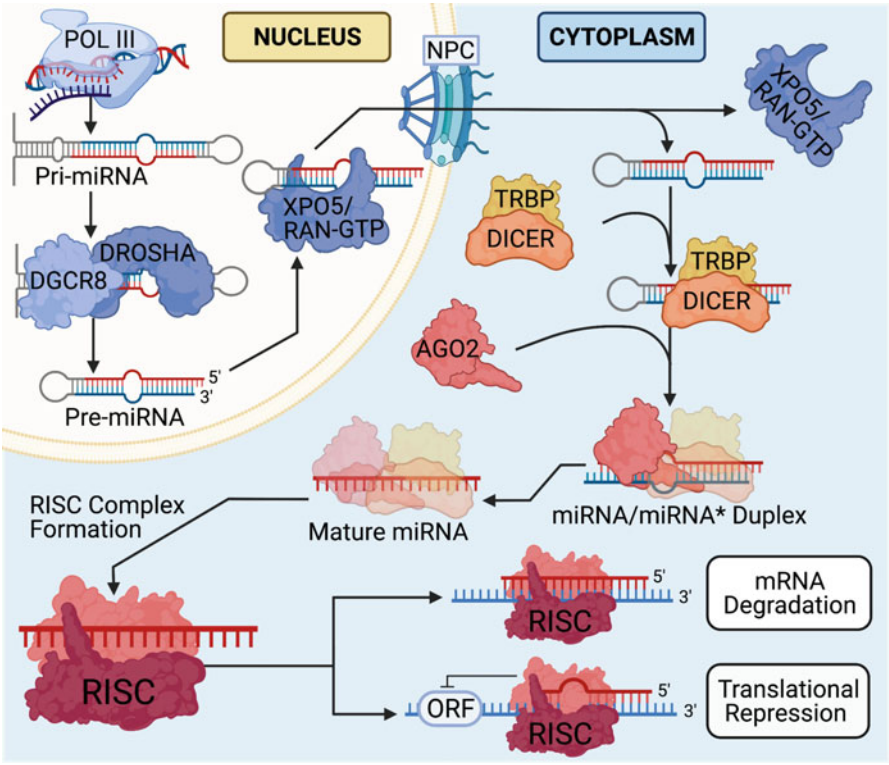

Fig. 2 miRNA biogenesis and mechanisms of gene expression. RNA polymerase III (POL III), primary miRNA (pri-miRNA), DiGeorge Syndrome Critical Region 8 (DGCR8), precursor miRNA (pre-miRNA), exportin-5 (XPO5), nuclear pore complex (NPC), transactivation response RNA binding protein (TRBP), argonaute-2 (AGO2), RNA-induced silencing complex (RISC), open reading frame (ORF)

microprocessor complex to form ~70-nucleotide hairpin RNA precursor miRNAs (pre-miRNAs).The pre-miRNA is transported out of the nucleus, through a nuclear pore complex (NPC), into the cytoplasm by the exportin-5 (XPO5)/RAN-GTP complex to become cleaved into a double-stranded miRNA/miRNA* duplex by the endoribonuclease Dicer and transactivation response RNA binding protein (TRBP) (Chendrimada et al. 2005). The miRNA/miRNA* duplex is integrated into an argonaute-2 (AGO2) protein and is unwound by helicase into two single-stranded miRNAs. Other proteins then bind to Dicer, TRBP, and AGO2 to complete the RNA-induced silencing complex (RISC). The mature miRNA then binds to the 3' UTR of its target mRNA, upstream of the open reading frame (ORF), and utilizes its inhibitory function via translational repression or degradation of the mRNA. Translational repression or transcript degradation typically, but not exclusively, depends on the degree of base complementarity between a miRNA and its target mRNA (Oliveto et al. 2017). If the miRNA binds with high complementarity, the

mRNA transcript will be degraded. Alternatively, if the miRNA binds with lower complementarity, translation will be disrupted. In addition to translation repression, miRNAs binding to their target mRNAs promote mRNA decay factors that cause mRNA destabilization and degradation, resulting in decreased expression levels of the target. Aberrant miRNA expression is associated with various human diseases, including breast cancer, and miRNAs regulate different cellular pathways in breast cancer progression and metastasis.

miRNAs in Breast Cancer

Roughly 50% of human miRNA-encoding genes are in the region of chromosomes where most driver mutations, cancer-associated regions, or fragile chromosome sites are located (Loh et al. 2019). This has led to the discovery of numerous miRNAs and their respective dysregulation in different types of cancer, including breast cancer (Oliveto et al. 2017; Loh et al. 2019). The miRCancer database contains an updated list of 324 unique miRNAs in breast cancer, identified from 632 different publications (Xie et al. 2013).

miRNAs can be either oncogenic or tumor-suppressive and control various cellular pathways of breast cancer progression such as cell proliferation, apoptotic response, metastasis, cancer recurrence, and chemoresistance (Oliveto et al. 2017; Loh et al. 2019). Oncogenic miRNAs are usually upregulated in breast cancer and suppress the expression of tumor suppressor genes or stimulate proliferation and cell survival. In contrast, tumor suppressor miRNAs are generally downregulated in breast cancer, promoting breast cancer progression.

For example, the overexpression of two oncogenic miRNAs, miR526b and miR655, in poorly metastatic breast cancer cell lines MCF7 and SKBR3 advances cell growth, migration, invasion, epithelial to mesenchymal transition (EMT), cancer stem cell (CSC) accumulation, oxidative stress, angiogenesis, and lymphangiogenesis in vitro (Majumder et al. 2015; Majumder et al. 2018a; Shin et al. 2019; Hunter et al. 2019). miR526b and miR655's roles in EMT, cell migration, vascular mimicry, angiogenesis, and oxidative stress are promoted further in hypoxic conditions (low oxygen levels) (Gervin et al. 2020). Thus, both miR526b and miR655 positively regulate the TME in favor of tumor metastasis. miR526b and miR655 belong to different miRNA clusters consisting of 46 and 42 miRNAs in each cluster, respectively. miR526b belongs to a cluster of miRNAs found on chromosome 19 within the miR515 gene family, and miR655 is in a cluster of miRNAs on chromosome 14 in the miR154 gene family.

The TME

The TME is a highly heterogeneous environment consisting of tumor cells, endothelial cells, cancer-associated fibroblasts, macrophages, cell metabolites, extracellular matrix, growth factors, inflammatory molecules, chemokines, cytokines,

reactive oxygen species (ROS), cancer stem cell (CSC)s, and miRNAs (Whiteside 2008). Complex interactions occur within the TME and are mediated by secreted proteins, metabolites, and cell-to-cell signaling. The collection of proteins secreted by tumor cells, known as the secretome, makes up 13–20% of the proteome and can influence functions, such as oxidative stress, angiogenesis, lymphangiogenesis, and upregulation of CSCs within the TME (Mukherjee and Mani 2013). Furthermore, conditions within the TME differ from conventional physiological conditions, including increased hypoxia, acidity, and interstitial fluid pressure, all of which can alter the TME (Hui and Chen 2015).

Aggressive tumor cells also release inflammatory proteins, growth factors, ROS, superoxides, and miRNAs into the TME, acting as a double-edged sword where both tumor and neighboring cells in the TME positively interact to enhance tumor growth and invasion and reduce apoptotic signals (Whiteside 2008; Hui and Chen 2015). For instance, overproduction of the extracellular inflammatory signaling molecule prostaglandin E2 (PGE2) alters the TME, promoting angiogenesis, lymphangiogenesis, inflammation, stem (-like) cells formation, and tumor immune evasion (Majumder et al. 2018b; Lala et al. 2018; Hunter et al. 2019; De Paz Linares et al. 2021).

PGE2 signals can promote tumor metastasis via both paracrine and autocrine regulation. PGE2 can bind to four G-protein-coupled receptors, PGE2 receptor (EP) 1–4. Both EP2 and EP4 facilitate protein kinase A (PKA) pathway activity, while EP4 also activates the noncanonical phosphoinositide 3-kinases/protein kinase B (PI3K/AKT) and extracellular signal-regulated kinase (ERK) pathways (Majumder et al. 2018b; Lala et al. 2018). Activation of EP2 and EP4 receptors is most notable for promoting angiogenesis and lymphangiogenesis by stimulating the release of vascular endothelial growth factors (VEGFs) and chemokines while also mediating various immune responses (Xin et al. 2012; Hunter et al. 2019). In breast cancer, the overproduction of PGE2 and its subsequent release into the TME facilitate tumor-associated angiogenesis, lymphangiogenesis, CSC formation, and tumor immune evasion through EP2 and EP4 receptor activation (De Paz Linares et al. 2021).

COX-2 and miRNA both positively regulate each other in breast cancer. The overexpression of COX-2 in the poorly metastatic luminal A breast cancer cell line MCF7 upregulates two oncogenic miRNAs, miR526b and miR655, and similarly, miR526b- and miR655-overexpressed breast cancer cell lines showed high expression of COX-2 (Majumder et al. 2015, 2018a). The increased COX-2 expression in these miRNA overexpressing cell lines enhances EP4 receptor activity, accounting for the aggressive cancer phenotypes mediated by these miRNAs, which will be discussed in detail in the following sections of this chapter.

Hypoxia and Oxidative Stress

Hypoxia plays a critical role in the TME in breast cancer. In cancer, hypoxia results from cancer cells outgrowing their available blood supply, limiting oxygen

availability to the middle of an aggressively growing tumor. Most solid tumors have some level of hypoxia, which is associated with many cancer hallmarks and contributes to poor patient outcomes (Choudhry and Harris 2018). In response to hypoxia, cancer cells secrete various molecules, stimulating ROS production, tumor-associated angiogenesis and lymphangiogenesis, CSC induction and maintenance, and inflammation (Yang et al. 2020; Gervin et al. 2020).

Hypoxia initiates numerous signaling pathways, including hypoxia-inducible factor (HIF), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), ERK, and phosphoinositide 3-kinases/protein kinase B/mechanistic target of rapamycin (PI3K/AKT/mTOR) pathways, which participate in proliferation, migration, inflammation, and anti-apoptotic functions in cancer (Muz et al. 2015). Hypoxic cells counteract oxidative stress by different transcriptional and posttranscriptional methods, prominently regulated by HIF-1, a transcription factor in charge of oxygen homeostasis and metabolic activity (Choudhry and Harris 2018). The HIF-1 complex consists of hypoxia-inducible factor 1 alpha (HIF-1α) and hypoxia-inducible factor 1 beta (HIF-1β) subunits. In normal oxygen conditions (normoxia), both subunits are actively transcribed; however, the HIF-1 complex cannot form due to HIF-1α degradation in the presence of oxygen. This occurs as an oxygen-dependent degradation domain of the HIF-1α subunit is hydroxylated by enzymes prolyl hydroxylase domain (PHD) and factor inhibiting HIF-1 (FIH-1), which allows tumor suppressor von Hippel-Lindau (pVHL) to catalyze the ubiquitination of HIF-1α. In hypoxia, HIF-1α degradation is averted, and HIF-1 complex forms. HIF-1α stabilization can have implications in hypoxic conditions within the TME of a growing tumor, potentiating tumor-associated angiogenesis and lymphangiogenesis, ROS production, and CSC formation and maintenance (Yang et al. 2020).

Furthermore, there is a complex dynamic between hypoxia and ROS generation, as hypoxic conditions increase ROS and oxidative stress levels (McGarry et al. 2018). Hypoxia-induced ROS overproduction stimulates the oxidation of biological molecules, such as DNA, lipids, and proteins. HIF increase and ROS formation happen concurrently in hypoxia (Chen et al. 2018). ROS increases HIF accumulation during hypoxia, while HIF accumulation during hypoxia can promote or hinder ROS formation through downstream transcriptional targets. These stringent conditions allow mutations to occur, and in a perfect enriched environment like the TME, clonal expansion of niche cells with stem (-like) phenotypes is selected, contributing to therapeutic resistance (Yang et al. 2018). In cancer cells, increased ROS through HIF-1α can regulate gene expression associated with glycolysis. In non-hypoxic conditions, ROS can stabilize HIF-1α, advancing glycolysis, therefore allowing tumor cells to continue to proliferate. Alternatively, preventing ROS production during hypoxia decreases HIF-1α stabilization and subsequent expression of HIF-1α regulated genes (Weinberg and Chandel 2009). Although the mechanisms between hypoxia and ROS are not fully understood, some ROS may inhibit PHD and FIH hydroxylase activity, preventing HIF-1α inactivation.

miRNAs Regulating Hallmarks of Cancer

More recently, research focus has turned toward the mechanism by which miRNAs alter the TME in favor of breast cancer progression. miRNA dysregulation enhances many breast cancer phenotypes in the TME, including tumorigenesis, tumor growth, cell proliferation, metastasis, migration, invasion, drug resistance, oxidative stress, angiogenesis, lymphangiogenesis, and CSCs (Fig. 3). Tumor-secretory miRNA have also been found in the TME (exosomal miRNA), indicating miRNAs' ability to modulate cellular activity within the TME directly. These miRNAs include miR100, miR526b, and miR655 regulating tumorigenesis; miR30a, miR105, and miR510 benefiting tumor growth; miR34a, miR122, and miR124 inducing cell proliferation; miR204, miR526b, and miR655 stimulating angiogenesis; miR19b, miR22, and miR203 advancing metastasis; miR7a, miR526b, and miR655 supporting migration; miR10b, miR526b, and miR655 increasing invasion; miR34a, miR526b, and miR655 promoting CSCs; miR129, miR145, and miR1246 aiding in drug resistance; and miR204, miR526b, and miR655 controlling oxidative stress (Majumder et al. 2015, 2018a; Shin et al. 2019; Hunter et al. 2019; Liu et al. 2019; Xie et al. 2013). miRNAs are also involved in response to hypoxia, promoting the expression of HIF-1α and VEGFA, further enhancing tumor survival. Many miRNA expressions, including miR23, miR24, miR26, miR27, miR103, miR107, miR181, miR210,

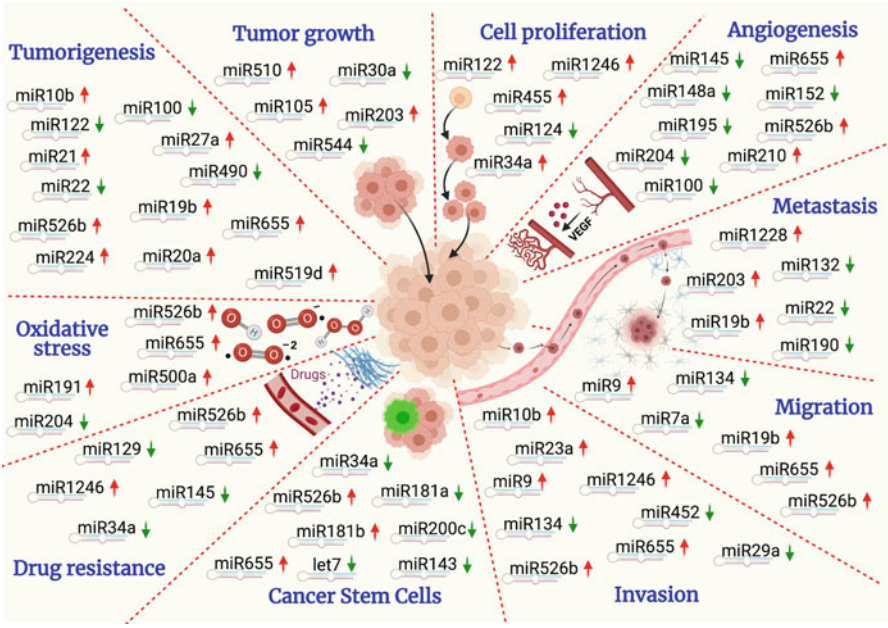

Fig. 3 miRNA and their regulatory functions within the breast cancer TME. Red arrows indicate that the upregulation of miRNA is enhancing the function. The green arrow indicates that the downregulation of miRNA is promoting the function

miR213, miR526b, and miR655, are promoted in hypoxic conditions via the induction of HIF-1α expression and desensitize tumors to pro-apoptotic signals (Kulshreshtha et al. 2007; Gervin et al. 2020). Therefore, understanding the interactions between miRNA in the breast cancer TME can be advantageous to diagnostics and therapeutics for patients.

Oxidative Stress Regulating the TME

Under standard physical conditions, ROS like hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hydroxide (OH^-), hypochlorous acid (HOCl), peroxyl radicals (ROO·), and superoxide (SO) form during the biosynthesis of macromolecules, metabolism, and respiration. The overproduction of ROS induces inflammation, dysregulates the cell cycle, and can stimulate intracellular transduction pathways (Federico et al. 2007). SO production results as a normal consequence of oxidative phosphorylation and behaves as an active signaling molecule regulating cell survival and proliferation. In standard physiological systems, there is a homeostatic balance between macromolecules and ROS. The inability of a cell to efficiently eliminate and neutralize excess ROS results in oxidative stress, which can induce damage to different cellular processes and organelles, altering normal physiology. In tumors, cancer cells can alter oxidative metabolism and signaling pathways, leading to increased ROS levels. This elevation in ROS increases the risk of oxidative damage and, therefore, causes mutations (Weinberg and Chandel 2009; Yang et al. 2018). Mutations can lead to the dysregulation of tumor suppressors and oncogenes and regulation of noncoding RNA, further promoting cancer progression.

Antioxidants are stable molecules that donate electrons to neutralize free radicals and serve as a natural defense mechanism against oxidative stress. Various enzymes regulate cellular detoxification pathways to remove ROS, such as superoxide dismutase, catalase, glutathione peroxidase, cysteine residues, and thioredoxin (TXN). TXN is an ubiquitous antioxidant protein that functions as a dithiol and disulfide balance regulator (Arnér and Holmgren 2000). Thioredoxin reductase 1 (TXNRD1) is the protein responsible for converting TXN into its active state, allowing TXN to neutralize products of oxidative stress. Overexpression of *TXNRD1* is associated with an increase in oxidative stress and poor survival in breast cancer patients (Leone et al. 2017). Elevated ROS also activates numerous other transcription factors, including nuclear factor erythroid 2-like 2 (NFE2L2), a transcription factor targeting multiple antioxidant genes such as *TXNRD1*, *HIF-1*, and *NFκB* (Priya Dharshini et al. 2020). HIF-1 is associated with the cellular hypoxic response pathway and also mediates ROS levels by transcribing genes, such as *heme oxygenase-1 (HO-1)*, *inducible nitric oxide synthase (iNOS)*, and *endothelial nitric oxide synthase (eNOS)*, to mitigate intracellular ROS levels. Intracellular ROS also activates the NFκB transcription pathway, producing inflammatory cytokines and chemokines while also preventing apoptosis (Babu and Tay 2019). miRNAs can regulate oxidative stress directly or indirectly by targeting genes associated with

TXNRD1 production, by modulating intracellular ROS, or by targeting *TXNRD1* transcription factors, altering ROS within the TME (Shin et al. 2019).

Oxidative Stress and miRNAs

Many miRNAs have connections with oxidative stress pathways in breast cancer and their regulation in the breast cancer TME (Fig. 3). miR204 was found downregulated in all breast cancer subtypes and directly targets phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (PIK3CB), a catalytic domain of the PI3K kinases and therefore a major regulator of the PI3K/AKT pathway (Hong et al. 2019). miR204 overexpression abrogates cancer metabolism and cell proliferation by disrupting PI3K kinase activity, thereby reducing intracellular ROS production. miR500a directly regulates the oxidative stress response genes *TXNRD1* and *NFE2L2* in MCF7 cells and is correlated with breast cancer progression and cell survival, indicating that miR500a is an oxidative stress response miRNA in breast cancer (Degli Esposti et al. 2017). The overexpression of miR21 results in increased tumorigenesis and promotes anti-apoptotic signaling in lung, liver, prostate, ovarian, and breast cancers (Krichevsky and Gabriely 2009). A target of miR21 is the transcript for mammary serine protease inhibitor (maspin), a pro-apoptotic protein shown to be critical in reducing ROS accumulation (Yin et al. 2005). In vitro, maspin overexpression stimulates apoptotic signaling in breast cancer cells, likely sensitizing these cells to oxidative stress signals (Liu et al. 2004).

In miR526b- and miR655-overexpressing MCF7 cells, both miRNAs were found to directly increase total and cellular ROS and SO levels and the expression of oxidative stress marker *TXNRD1* in comparison to miRNA-low MCF7 cells (Shin et al. 2019) (Fig. 4). miR526b and miR655 target two transcription factors, polybromo 1 *(PBRM1)* and transcription factor 21 *(TCF21)*, which are negative regulators of *TXNRD1,* leading to *TXNRD1* upregulation. Additionally, oxidative stress induces miR526b and miR655 expression, indicating a positive feedback loop between miR526b, miR655, and oxidative stress in breast cancer. Treatments with miRNA-high cell secretions enhanced ROS and SO production by MCF7 cells, as well as primary human umbilical vein endothelial cells (HUVECs), and cell-free miR526b and miR655 can be detected in the respective conditioned media, strongly suggesting that miRNAs are involved in extracellular signaling and regulating oxidative stress in the TME. A subsequent study showed that hypoxic conditions further promote ROS and SO generation in miR526b- and miR655-overexpressing breast cancer cell lines (Gervin et al. 2020). The addition of a COX-2 inhibitor (COX-2I, NS398), EP4 antagonist (EP4A, ONO-AE3-208), or PI3K/AKT pathway inhibitor (WM, Wortmannin) reduced ROS production in both MCF7-miR526b and MCF7-miR655, altogether displaying that hypoxia enhances oxidative stress in miRNA-high cells and that ROS generation in miR526b- and miR655-high breast cancer cells is dependent on COX-2, EP4, and PI3K/AKT pathways.

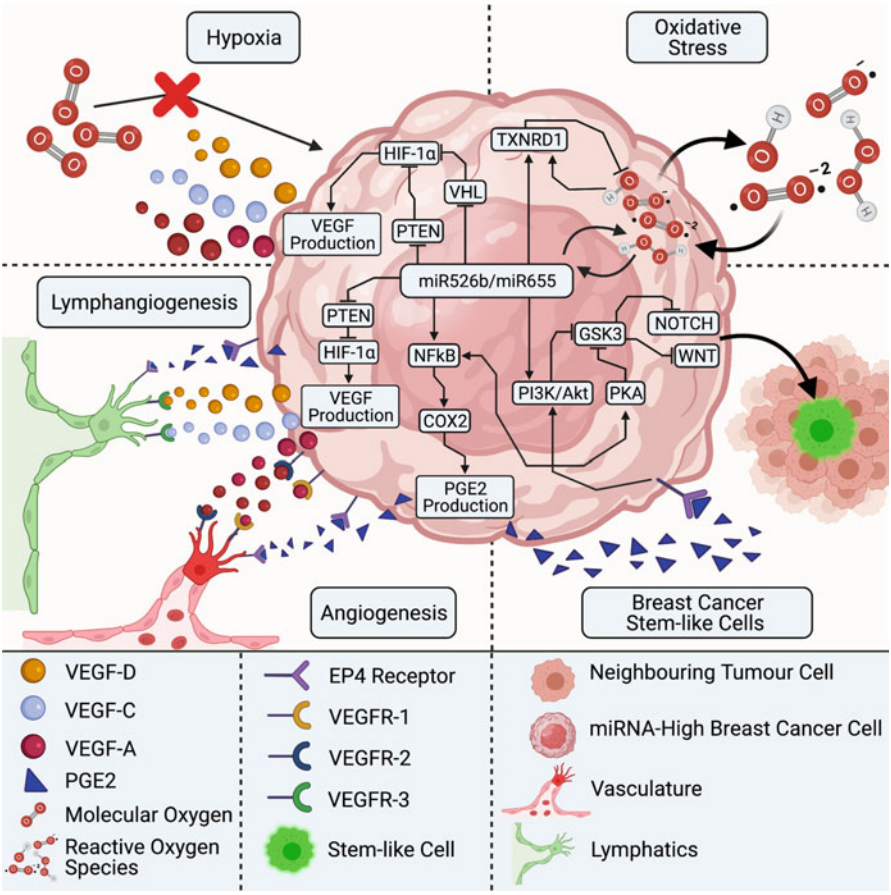

Fig. 4 The proposed mechanisms and functions of miR526b and miR655 in the breast cancer TME

Tumor-Associated Angiogenesis and Lymphangiogenesis

Angiogenesis and lymphangiogenesis are essential biological functions that aid in development, reproduction, and wound repair. Angiogenesis is the process by which new blood vessels are produced from preexisting vasculature, allowing oxygen and nutrient delivery to cells (Otrock et al. 2007). In comparison, lymphangiogenesis is how new lymphatic vessels form from preexisting lymphatic vessels, allowing the circulation of immune cells throughout the body and maintaining interstitial fluid levels. In a growing tumor, growth factors are released, such as VEGFA, VEGFC, and VEGFD, into the TME to stimulate the growth and migration of endothelial cells carrying receptors for these ligands. Angiogenic factor VEGFA binds to VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2), and lymphangiogenic factors VEGFC and VEGFD bind to VEGF receptor 3 (VEGFR3). Both tumor cells and tumor-infiltrated immune cells release VEGFs to enhance angiogenesis and

lymphangiogenesis (Xin et al. 2012; Majumder et al. 2014) Tumor cells also release PGE2, which binds to EP receptors to enhance tumor growth (De Paz Linares et al. 2021). The release of inflammatory cytokines, chemokines, and signaling molecules can initiate angiogenesis and lymphangiogenesis (Otrock et al. 2007). COX-2-high breast cancer cells also release chemokines in the TME, promoting lymphangiogenesis. Tumor cells release chemokines, such as CCL21 which binds to CCR7 on lymphatic endothelial cells, promoting lymphangiogenesis and lymph node metastasis. *CCL21* expression in human breast cancer is positively correlated with the expression of lymphangiogenic markers *VEGFC*, *LYVE1*, and *PROX1* (Tutunea-Fatan et al. 2015). A growing tumor also becomes hypoxic and further enhances the HIF-1 hypoxic response pathway, leading to greater pro-angiogenic and pro-lymphangiogenic signaling in hypoxia (Morfoisse et al. 2015).

Oxidative Stress and Tumor-Associated Angiogenesis

In the vasculature, high ROS levels have a negative impact on most tissue types (Kim and Byzova 2014). In comparison, low ROS levels can activate different signaling pathways that promote growth and regeneration. In endothelial cells, transient levels of ROS are essential for VEGFA-mediated angiogenesis. Thus, ROS can be detrimental directly and indirectly in angiogenic functions. Oxidative stress associated with angiogenesis is a prominent participant in cancer progression. In a growing tumor, oxidative stress plays a beneficial role during tumor-associated angiogenesis, despite the damaging effects on tissues at high concentrations. Several studies have demonstrated this positive interrelationship between ROS and angiogenesis. For example, treatments with exogenous ROS sources, such as H_2O_2, promote VEGFA production by tumor cells. ROS-mediated angiogenesis enhances VEGFA expression; similarly, VEGFs further stimulate ROS production through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in endothelial cells. ROS produced through NADPH oxidase activation promotes the autophosphorylation of VEGFA receptor VEGFR2, indicating the importance of ROS as signaling molecules in angiogenesis.

Roles of miRNAs in Angiogenesis and Lymphangiogenesis in Breast Cancer

In cancer, miRNA dysregulation can alter angiogenic functions in the TME, caused by many factors, including the activation of inflammatory signaling molecules or pro-angiogenic growth factors (Liu et al. 2019). Exosomal miR210, secreted by breast cancer cells, advances angiogenic functions in co-cultured endothelial cells by allowing VEGFA expression by targeting mRNA transcripts for proteins ephrin-A3 and tyrosine phosphatase 1B (PTP1B), and these effects were enhanced in hypoxic conditions (Jung et al. 2016). Alternatively, mesenchymal stem cell-derived exosomal miR100 secretions decreased VEGFA expression and its subsequent release from breast cancer cell lines (Pakravan et al. 2017). These effects are mediated by miR100 directly targeting mTOR and therefore blocking the activity of the mTOR/HIF-1α/VEGF signaling axis, leading to the inhibition of endothelial angiogenic behavior. miRNAs involved in angiogenesis are summarized in Fig. 3.

miR526b and miR655 can regulate angiogenesis and lymphangiogenesis by COX-2 overexpression and EP4 activation (Hunter et al. 2019) or indirectly by targeting genes or transcription factors associated with angiogenesis and lymphangiogenesis (Fig. 4). In miR526b- and miR655-high cell lines, COX-2 expression is upregulated via the NFκB pathway as both miRNAs target known inhibitors of the NFκB pathway (Majumder et al. 2015, 2018a). miR526b targets *Ras1* (ras-like protein 1) and miR655 targets both ras-related protein rab-7 L1 (*RAB7L1*) and tumor protein 53-induced nuclear protein 1 (*TP53NP1*), enhancing COX-2 expression, EP4 receptor activity, and therefore ERK and PI3K/AKT signaling. Moreover, stimulation of the ERK and PI3K/AKT pathways indirectly enhances miR526b and miR655 expressions. miR526b and miR655 indirectly regulate the expression of tumor suppressors *PTEN* and *VHL* (Hunter et al. 2019; Gervin et al. 2020). PTEN blocks the PI3K/AKT signaling pathway, and pVHL inhibits HIF-1α activity. Thus, by targeting these two tumor suppressor genes, miR526b and miR655 promote angiogenic functions within breast cancer cells and the breast cancer TME.

miR526b and miR655 expressions in human breast cancer tissues are positively correlated with angiogenic markers *VEGFA* and *CD31*, and lymphangiogenic markers *LYVE1*, *VEGFC*, and *VEGFD*, indicating the roles of miRNA in regulating angiogenesis, lymphangiogenesis, and metastasis (Hunter et al. 2019). Endothelial cells make up the lining of blood vessels and carry EP4 receptors and the VEGF receptors VEGFR1 and VEGFR2, and treating HUVECs with MCF7-miR526b and MCF7-miR655 cell-free conditioned media increased *VEGFR1* and *VEGFR2* and *EP4* gene expression (Fig. 4). Cell-free conditioned media from miR526b- and miR655-high breast cancer cells also enhanced cell migration and tube formation in HUVEC cells compared to treatment with miRNA-low conditioned media. Hypoxic conditions further promoted migration and tube formation in miRNA-high breast cancer cells, and the addition of COX-2I, EP4A, or WM abrogated migration and tube formation (Hunter et al. 2019; Gervin et al. 2020).

Furthermore, *NFκB1*, *COX-2*, and *VEGFA* gene expressions were significantly higher in hypoxic conditions for MCF7-miRNA-high cells compared to parental MCF7 cells (Gervin et al. 2020). Hypoxic conditions further enhanced *EP4* expression in breast cancer cells, indicating that EP4 signaling is essential for tumor cells to survive in hypoxia. *VHL* gene expression was significantly lower in miR526b- and miR655-overexpressing breast cancer cell lines, resulting in elevated HIF-1α protein levels in normoxic and hypoxic conditions. Increased HIF-1α protein and gene expressions and decreased *VHL* gene expression in miR526b- and miR655-high breast cancer cells under hypoxic conditions indicate that hypoxia further enhances the pro-angiogenic functions of MCF7-miR526b and MCF7-miR655 breast cancer cells (Fig. 4). Treatment with selective inhibitors, COX-2I and EP4A, abrogated *HIF-1α* gene expression in hypoxic conditions in miRNA-high breast cancer cells. Collectively, this demonstrates that miR526b, miR655, and secretions from miRNA-high tumors promote angiogenic and lymphangiogenic functions via the COX-2, EP4, and PI3K/AKT signaling pathways in breast cancer, which is enhanced in hypoxic conditions.

CSCs and Oxidative Stress

CSCs are a small subset of cells within tumors that can promote tumor induction, metastasis, modulation of the TME, and resistance to cancer therapies (Wicha et al. 2006; Yang et al. 2020). This group of cells is different from other malignant cells within a tumor due to their self-renewal properties and capability to generate progenitor cells that can take various differentiation paths, adding to the heterogeneous composition of solid tumors. The overexpression of aldehyde dehydrogenase (ALDH) or cell surface marker cluster of differentiation 44 (CD44) and reduced levels of cell surface marker cluster of differentiation 24 (CD24) are commonly known and validated as functional breast cancer stem cell markers (Yang et al. 2015; Majumder et al. 2015, 2018a). Specific transcription factors, such as octamer-binding transcription factor 3/4 (OCT3/4), SRY-box transcription factor 2 (SOX2), Nanog homeobox (NANOG), and MYC proto-oncogene (MYC), and cell signaling and stem cell pathways, including the PI3K/AKT, NFκB, Notch, and Wnt, are fundamental to conventional stem cell operations and are often dysregulated in cancer and regulate CSCs (Majumder et al. 2015, 2018a; Yang et al. 2020). Taking advantage of CSC properties, a subpopulation of cells within a tumor can resist traditional therapies, ultimately promoting cancer relapse.

There is a complex interaction between CSCs and the TME. For example, the fusion of mesenchymal stem cells with tumor cells increases heterogeneity and alters cancer cell functions in the TME (Yang et al. 2015). Physical conditions within the TME can directly regulate CSCs, as hypoxic conditions select for CSC phenotypes, such as spheroid formation in tumor cell populations. Many CSCs, including breast CSCs, contain lower levels of ROS than their non-CSC counterparts, likely due to the expression of oxidative stress response genes, allowing therapeutic resistance (Diehn et al. 2009; Shi et al. 2012; Prager et al. 2019). Furthermore, inhibition of ROS scavengers within CSC populations may increase CSC ROS levels, and research suggests that this could reduce clonogenicity and promote differentiation, thereby preventing CSC therapeutic resistance. The secretions of inflammatory-associated molecules within the TME, such as PGE2, from both tumor and tumor-infiltrating immune cells, encourage CSC formation (Yang et al. 2020). Therefore, deciphering the relationship between CSCs and the TME is necessary for understanding tumor growth and therapeutic resistance.

CSCs and miRNAs

miRNAs play a major role in the regulation of CSC functions and phenotypes in breast cancer (Majumder et al. 2015, 2018a; Liu et al. 2019). For example, the Let7 miRNA family and miR200c are inversely associated with the CSC phenotype in human breast cancer (Yu et al. 2007; Shimono et al. 2009; Sun et al. 2014). The expression of Let7 family miRNAs was associated with reduced self-renewal and upregulation of CSC differentiation in breast cancer cells by targeting hras proto-oncogene (HRAS) and high mobility group AT-hook 2 (HMGA2) genes (Yu et al. 2007). Additionally, miR200c was one of many miRNAs shown to be down-regulated in CSCs, and one of the miR200c targets is BMI1 proto-oncogene

(BMI1), a proto-oncogene implicated in stem cell self-renewal (Shimono et al. 2009). In normal mammary stem cells, miR200c expression prevents differentiation and duct formation. In cancer, miR200c suppressed the proliferation of embryonal carcinoma cells (malignant counterparts of embryonic stem cells) and the clonal expansion of breast cancer cells in vitro, as well as tumor development directed by breast CSCs in vivo. miRNAs regulating CSCs are highlighted in Fig. 3.

miR526b and miR655 were found to directly regulate CSC phenotypes via the Notch/Wnt pathway (Majumder et al. 2015, 2016, 2018a) (Fig. 4). Overexpression of miR526b and miR655 in MCF7 and SKBR3 cell lines leads to increased CSC populations, and miR526b- and miR655-overexpressing MCF7 and SKBR3 cells displayed higher spheroid formation efficiency levels compared to their parental cell lines (Majumder et al. 2015, 2018a). Knockdown of either miRNA could disrupt spheroid formation and CSC phenotypes of aggressive ER-positive COX-2-high breast cancer cells. The overexpression of miR655 generated an increase in *Notch* gene expression and the expression of Wnt-associated genes, such as *MYC*, *cyclin D1 (CCND1)*, and *axis inhibition protein 2 (AXIN2)* (Majumder et al. 2018a). Furthermore, miR655 overexpression elevated protein levels of CSC marker ALDH and pluripotency marker OCT3/4, strongly supporting the involvement of miR655-induced CSC phenotypes in breast cancer. Spheroid-forming subpopulations of poorly metastatic breast cancer cell lines showed higher expression of both miR526b and miR655. This was observed when COX-2 was overexpressed in the poorly metastatic breast cancer cell lines MCF7 and SKBR3, but also when poorly metastatic MCF7 and T47D breast cancer cells were treated with EP4 agonist, PGE1OH (Majumder et al. 2016). EP4 signaling perturbs glycogen synthase kinase-3 (GSK3), a negative regulator of the Notch and Wnt pathways, leading to increased Notch and Wnt pathway signaling. In the future, it would be interesting to examine if oxidative stress enhances CSCs in miRNA-high tumors, relating chemotherapy, drug resistance, and miRNA involvement in CSC induction.

Drug-related toxicity and chemotherapy cause overproduction of treatment-resistant CSC population. In vitro treatments of aggressive breast cancer cells with COX-2I and EP4A significantly abrogated CSCs, and also EP4 knockdown in MCF7-COX-2 inhibited tumor metastasis (Majumder et al. 2015, 2018a). EP4A is an ideal drug to reduce major miRNA-induced aggressive breast cancer phenotypes and shows no toxicity in vitro and in vivo and effectively reduces CSCs, angiogenesis, lymphangiogenesis, and lung and lymph node metastasis (Xin et al. 2012; Majumder et al. 2014; 2016).

miRNA Therapeutic Relevance in Cancer

While few miRNAs have been studied in clinical trials as cancer therapeutics, especially for breast cancer, many miRNAs show promise in preclinical settings (Rupaimoole and Slack 2017). The dysregulation of many miRNAs presents avenues for possible therapeutic intervention in cancer by directly restoring tumor-suppressive miRNAs using miRNA mimics, by suppressing oncogenic miRNAs

using anti-miRNAs, or by exploiting the tumor-promoting pathways that these miRNAs regulate. Different strategies have been implemented to revive miRNA with tumor-suppressive properties using miRNA mimics. miRNA mimics can be chemically modified to have direct delivery to tumors through local injection or systemic approaches, while other delivery models are still under investigation, such as using viral vectors or lipid-based nanoparticles to enhance miRNA delivery. Many miRNAs have shown the ability to restore tumor-suppressive properties, including miR16, miR26a, miR34, miR200, miR506, and miR520. Alternatively, various trials have evaluated methods to suppress oncogenic miRNAs miR10b, miR155, miR221, and miR630 by using anti-miRNAs, to inhibit various tumorigenic processes. Currently, miR16 and miR34 are in phase I clinical trials for lung cancer and liver cancer respectively, while many other miRNAs present potential preclinical results.

Two miRNAs that show encouraging preclinical findings are the overexpression of miR526b and miR655 for therapeutic intervention (Majumder et al. 2015, 2018a). These miRNAs rely greatly on COX-2 activity and EP4 receptor pathways and promote many cancer phenotypes, including CSCs, tumor-associated angiogenesis, lymphangiogenesis, and oxidative stress (Majumder et al. 2015, 2018a; Shin et al. 2019; Hunter et al. 2019). Therefore, miR526b and miR655 could be indirectly exploited by blocking EP4 receptor activity by using a specific EP4 receptor antagonist. EP4 is already a drug target in chronic inflammatory diseases, like arthritis, and shows promise as a therapeutic target in various cancers, including breast cancer in clinical trials (De Paz Linares et al. 2021). miR526b and miR655 expressions are positively correlated with *COX-2* and *EP4* in human breast tumors, and the expression and function of these miRNA are abrogated with an EP4 antagonist in aggressive breast cancer cell lines. Thus, EP4 presents a promising therapeutic target for COX-2-, EP4- and miR526b/655-high breast cancer.

Biomarker Capability of miRNAs in Breast Cancer

Approximately 100 miRNAs have been identified in the blood, serum, or plasma of breast cancer patientsas potential diagnostic, prognostic, or predictive breast cancer biomarkers (Hamam et al. 2017; Majumder et al. 2021; Sohel 2020). Many of these miRNAs have been shown to play a prominent role in the TME regulation, including miR10b, miR21, miR34a, miR122, miR143, miR145, miR181b, miR195, miR210, miR500a, miR1246, miR526b, and miR655. For example, miR526b and miR655 cell-free secretions stimulate many important cancer-promoting phenotypes such as oxidative stress, angiogenesis, lymphangiogenesis, and induction of CSCs in the TME and they can be detected in the plasma of breast cancer patients (Majumder et al. 2015, 2018a, 2021; Shin et al. 2019; Hunter et al. 2019; Gervin et al. 2020). Additionally, bodily fluids near breast tumors contain secreted proteins and miRNAs, allowing cancer-specific secretory proteins to be detected in the blood and potentially be used as blood-based breast cancer biomarkers (Pavlou and Diamandis 2010; Mukherjee and Mani 2013). Few validated studies compare

tumor-specific circulating miRNA with their premature counterparts (pri-miRNAs) in breast cancer. Measuring pri-miRNA in the blood or plasma of breast cancer patients is considered beneficial because instead of a mature miRNA-specific cDNA synthesis and amplification, which requires high RNA concentration for each miRNA detection;pri-miRNA is easy to measure and quantify like any other gene in the blood with RNA extraction and global cDNA synthesis instead of each miRNA-specific cDNA synthesis, thus reducing the demand of large quantity of patient specimens. Pri-miR526b and pri-miR655 have similar levels of expressions compared to their respective mature miRNAs (miR526b and miR655) in patient blood plasma (Majumder et al. 2021). Both pri-miR526b and pri-miR655 showed increase in expression in the plasma of breast cancer patientplasma compared to benign plasma samples and pri-miR526b is a highly sensitive and specific biomarker to detect cancer as early as tumor stage I and could also differentiate early and late tumor stages. Pri-miR526b and pri-miR655 can provide opportunities to develop reliable blood-based biomarkers which can be used in conjunction with current screening protocols for early diagnosis of breast cancer. Pri-miRNAs are potential blood biomarker tools to develop a RNA-based breast cancer test that will provide minimally invasive breast cancer pre-screening options for younger populations and promote early detection.

Conclusion and Future Perspectives

It is well established that TME alterations can lead to adverse outcomes in cancer, and miRNAs are emerging factors that have shown to be critical regulators of the TME. The identification and characterization of miRNAs, such as miR526b and miR655, and their roles in aggressive breast tumors is promising since EP4A is a therapeutic target to abrogate these miRNA's functions in the TME. Also, the potential of miRNAs to serve as breast cancer biomarkers can improve disease outcomes by early detection. In the future, identification and validation of additional secretory proteins from miR526b- and miR655-high breast cancer cell secretions could identify novel therapeutic targets in breast cancer, and further validation of miRNAs and their corresponding secretory proteins may improve breast cancer diagnosis and therapeutic intervention.

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