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Review

Epithelial-to-Mesenchymal Transition Signaling Pathways Responsible for Breast Cancer Metastasis

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Abstract—Breast carcinoma is highly metastatic and invasive. Tumor metastasis is a convoluted and multistep process involving tumor cell disseminating from their primary site and migrating to the secondary organ. Epithelial-mesenchymal transition (EMT) is one of the crucial steps that initiate cell progression, invasion, and metastasis. During EMT, epithelial cells alter their molecular features and acquire a mesenchymal phenotype. The regulation of EMT is centered by several signaling pathways, including primary mediators TGF-β, Notch, *Wnt,* TNF-α, Hedgehog, and RTKs. It is also affected by hypoxia and microRNAs (miRNAs). All these pathways are the convergence on the transcriptional factors such as Snail, Slug, Twist, and ZEB1/2. In addition, a line of evidence suggested that EMT and cancer stem like cells (CSCs) are associated. EMT associated cancer stem cells display mesenchymal phenotypes and resist to chemotherapy or targeted therapy. In this review, we highlighted recent discoveries in these signaling pathways and their regulation in breast cancer metastasis and invasion. While the clinical relevance of EMT and breast cancers remains controversial, we speculated a convergent signaling network pivotal to elucidating the transition of epithelial to mesenchymal phenotypes and onset of metastasis of breast cancer cells.

Keywords—Breast cancer, EMT, Metastasis, Cancer stem cells, Cancer signaling pathways.

ABBREVIATIONS

AJ	Adherent junctions
AS	Alternative splicing
CSCs	Cancer stem cells
CT	Chemotherapy
EMT	Epithelial Mesenchymal Cell Transition
ER	Estrogen receptor
HER-2	Human epidermal growth factor receptor 2
miRNA	MicroRNA
PR	Progesterone receptor
TJ	Tight junctions
TGF	Transforming growth factor
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor

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INTRODUCTION

Breast carcinoma is one of the most common types of cancer in women. It is the fifth most common diseases leading to death worldwide. Invasion and metastasis are responsible for almost 90% of breast cancer death.[25] Molecular mechanisms underlying the cancer progression and metastasis have remained largely elusive.[23] Therefore, it is of paramount importance to understand these processes. Extensive efforts have been made to unveil the metastatic events in the last two decades.[122]

Tumor metastasis is a multistep process involving tumor cell disseminating from their primary site and migrating to the secondary organ.[59] It accompanies with a series of sequential steps, including cell invasion to a local tissue, intravasation to the blood circulation, transporting through vascularization, and extravasation to a secondary organ or tissue site.[13] Mounting evidence suggested that the epithelial-mesenchymal transition (EMT) plays a crucial role in promoting metastasis in breast carcinoma, although the actual mechanisms remain controversial.[128]

EMT is a convoluted biological event in which epithelial cells lose their adhesiveness ability and gain mesenchymal characteristics.[52] Three types of EMT have been discovered. Type 1 EMT is associated with embryogenesis, gastrulation, and neural formation; whereas type 2 is characterized by tissue regeneration and wound healing. Type 3 is linked to malignancy, invasion, and metastasis.[55] EMT is considered as one of the hallmarks of cancer invasion.[5] Morphologically, EMT program is characterized as an alteration in the phenotype from epithelial to mesenchymal cells.[60] The regulation of EMT mechanisms is ensured by several signaling pathways, including primary mediators TGF-β, Notch, and *Wnt*, and is also affected by hypoxia and expression of microRNAs (miRNAs).[91] All these pathways are the convergence on the transcriptional factors such as Snail, Slug, and Twist. Their expression leads to EMT.[31]

In this review, we first discussed breast cancer subtypes and their relationship with the EMT. We then highlighted EMT markers associated with breast cancer metastasis. Furthermore, we discussed signaling pathways involved in breast cancer EMT. In addition, we examined the effects of other factors such as hypoxia, miRNA, and alternative splicing on the breast cancer EMT. Finally, we stipulated the close relationship between EMT and the cancer stem cell and their impact on breast cancer metastasis.

HETEROGENEITY OF BREAST CANCERS AND EMT

Breast cancer is often a heterogeneous disease. It has long recognized that the diversity of cancer cells determines the progression of the cancer and its therapeutic resistance.[26] The molecular classification of breast cancers empowers a design of individual therapies. It will ultimately increase the survival rate.[21] It is, therefore, important to characterize the heterogeneity of breast cancer cells.

Subsets of breast cancer cells can be classified in several different ways. For instance, breast tumors can be categorized into six subgroups, i.e., normal-like, epidermal growth factor receptor (HER)-2$^+$, luminal A, luminal B, basal-like, and claudin-low, based on their gene expression profile.[17] The normal-like subgroup has a similar expression feature with non-cancerous breast cells. The HER-2 has an impact on several signaling pathways and is associated with dysregulated tumor growth, oncogenesis, metastasis and chemoresistance in breast cancer.[104] The overexpression of HER-2 is linked to a poor prognosis in chemo and targeted therapy.[22] The luminal A and B subgroups often express luminal cytokeratin 8/18 and estrogen receptor in a different manner.[28] The luminal A subgroup is associated with a higher estrogen receptor (ER) expression and a low HER-2 expression. The luminal B subgroup, however, is linked to a low ER expression and a high Ki67 index.[95] The basal-like subgroup is characterized with the expression of biomarkers, including cadherin, p63, cytokeratin 17, vimentin, cytokeratin 5/6 and cytokeratin in the basal myoepithelial cells of normal breast tissues.[92] The claudin-low subtype is associated by a low expression of cell-cell adhesion molecules, such as claudins 3, 4 and 7, occluding, and E-cadherin.[18] The claudin-low subtype is linked to the presence of EMT and stem-cell like features.[101] The basal-like and claudin-low subtypes are often found in triple-negative breast cancer (TNBC).[119] TNBC is characterized by the lack of PR, ER and HER-2 hormone receptors.[119] The over expression of estrogen and progesterone receptors are used as a predictive marker in hormone therapy.[88] These hormone receptors are used as an adjuvant endocrine therapy in the regulation of breast tumorigenesis.[88]

These six subsets of breast cancers and their association with EMT displays major differences. For instance, Luminal A and B are the most common breast cancer subtypes discovered in patients. These subtypes express ERα and ERα signals that suppress EMT.[117] It has been recognized that ERα expression in luminal A and B subtypes leads to a better prognosis as compared to TNBC subtypes. Ye, *et. al.* showed that ERα inhibits EMT via the suppression of Slug which leads to an increase in E-Cadherin expression.[142] Another study reported that ERα promotes stemness and EMT in breast cancers by suppressing BM1.[130] Therefore, ERα signaling is an essential regulating factor in luminal A and B subtypes that inhibit EMT in breast cancers.[130]

On the other hand, a number of studies suggested that a multipotent cytokine transforming growth factor β (TGF-β) can stimulate EMT in breast cancers. The TGF-β stimulation leads to an increase in Snail, TWIST, and ZEB 1/2 expression in the luminal A and B breast cancer cell lines.[146] The TGF-β-induced EMT activates EGFR-, IGF1R-, and MAPK-dependent ERα signaling and increase antiestrogen resistance.[146] Another pathway MEK-ERK is also associated with EMT in the luminal A and B subtypes of breast cancer cells.[118] MCF7 is a luminal cancer cell line. The VEGFR expression in MCF7 is associated with the expression of Snail and N-CADHERIN.[119] Taken together, it can be speculated that the ERα TGF-β, and MEK-ERK signaling pathways promote EMT in the luminal A and B subtypes.

EMT mechanisms of $HER2^+$ breast cancer cells are similar to those in luminal subtypes. They also undergo TGF-β-dependent EMT.[73] It was found that TGF-β–SMAD3 pathway is critical to EMT in $HER2^+$ cancers.[73] HER2 directly regulates the TGF-β expression and the activation of TGF-β/SMAD3 signaling. Additionally, the upregulation of transcription factors such as Slug and TWIST1 are crucial to EMT in HER^+ breast cancer subtypes.[78] One study showed that the AKT signaling activation increases the expression of Slug in $HER2^+$ breast cancer cells such as MDA-MB-453 and BT474.[78] Another regulator of TWIST1 is phosphorylated on Serine 68 residue in $HER2^+$ invasive ductal carcinomas.[10] It promotes breast cancer invasiveness.[43] Another study revealed that the overexpression of HER2 in MCF7 cells increased the expression of tumor kinase (Btk)/protein tyrosine kinase 6 (PTK-6) receptors that induce EMT and invasiveness.[2] Therefore, the TGF-β dependent pathways and TWIST and Slug transcription factors are crucial to EMT of $HER2^+$ breast cancer cells.

Another subtypes, TNBCs or basal like, claudin-low are the most aggressive breast cancer cells. Due to their lack of hormone-responsive receptors, they have limited therapeutic options.[139] TNBCs can be categorized into four groups; basal-like, mesenchymal, immunomodulatory, and luminal androgen receptor (AR)-positive subtypes.[139] The basal-like subtype of TNBC is associated with cell cycle and DNA damage pathways.[46] The ADP-ribose polymerase (PARP) inhibitors target to these pathways.[85] Mesenchymal TNBC tumors are associated with growth factor signaling such as PI3K/AKT and EMT that are inhibited by mTOR and eribulin mesylate.[74,96] Immunomodulatory TNBC are associated with immune cell signaling pathways such as NFκB and JAK/STAT pathways.[84] Therefore, immune checkpoint inhibitors could give a promising response to the patients with immunomodulatory TNBC. Luminal androgen receptor (AR^+) subtype tumors are associated with androgen-signaling and they can be treated by blocking the androgen receptor.[97] It has been discovered that the EMT of TNBCs is associated with the Notch, Hedgehog, TGF-β, and *Wnt* pathways. High Notch expression in TNBC patients is associated with poor survival rates.[36] The NUMB protein is used as an antagonist to Notch signaling to inhibit EMT in TNBC.[36,147] Notch signaling is important to the TNBC subtypes. More studies are needed to confirm the connection between Notch and EMT in TNBC, however.

Another hedgehog pathway is also critical to EMT in breast cancer.[8] It has been found that the hedgehog signaling activates three glioma-associated oncogenes, GLI1, 2, and 3 and GLI1. It is crucial to the EMT of breast cancer cells.[8] The hedgehog signaling is also associated with hypoxia-induced EMT invasiveness of MDA-MB-231 TNBC cells.[8] Like other types of breast cancer, TGF-β is also crucial to EMT and the stemness of MDA-MB-231 TNBC cells.[124] Okita, *et al.* showed that musculoaponeurotic fibrosarcoma (MAF) oncogene family protein K (MAFK) induces EMT in a TGF-β-dependent manner in TNBC cells.[82] It is suggested that TGF-β could be a hallmark regulator of EMT in these tumor cells. The *Wnt* signaling is another pathway that is crucial to TNBC metastasis. Upregulation of the *Wnt*/β-catenin signaling is associated with poor clinical outcomes in TNBC subtypes.[90]

Clearly, different breast cancer subsets display different behaviors under EMT. Breast cancer subtypes are regulated in different manners by EMT signaling pathways. Each pathway plays a different role in each subset. Therefore, targeting these pathways could be beneficial in terms of the drawback part of the breast cancer heterogeneity.

EPITHELIAL-MESENCHYMAL TRANSITION IN BREAST CANCER

The EMT is a natural and vital phenomenon in mesenchymal cell differentiation.[11] This process is essential for many biological processes, including wound healing, embryonic development, etc.[1] The correlation between EMT and cancer was first reported in the early 80s.[99] It was discovered that benign tumor cells acquired invasive properties after EMT. Since then, the role of EMT in metastasis of certain carcinogenic tumors has been described in various studies.[32] In lieu of increasing interest in understanding the relationship between EMT and cancer metastasis, the exact mechanism underlying EMT during cancer invasion and metastasis in breast cancer remains largely elusive. Nevertheless, signaling pathways involved in EMT and their associated transcription factors have been extensively investigated. Understanding these signaling pathways will help discover new therapeutic targets and the biomarkers for aggressive breast cancers.

Metastasis starts when tumor cells gain the ability to disseminate from their primary tumor site and invade into the surrounding tissues that are referred to as stromal tissues. The dissemination occurs either as a group or as single cells.[30] Epithelial tumor cells are usually connected with their surrounding cells through E-cadherin.[141] E-cadherin is an adhesion molecule present in adherent junctions (AJ) that connect epithelial cells tightly.[141] To metastasize, tumor cells break these intercellular junctions, migrate as single cells, and invade into neighboring tissues, as shown in

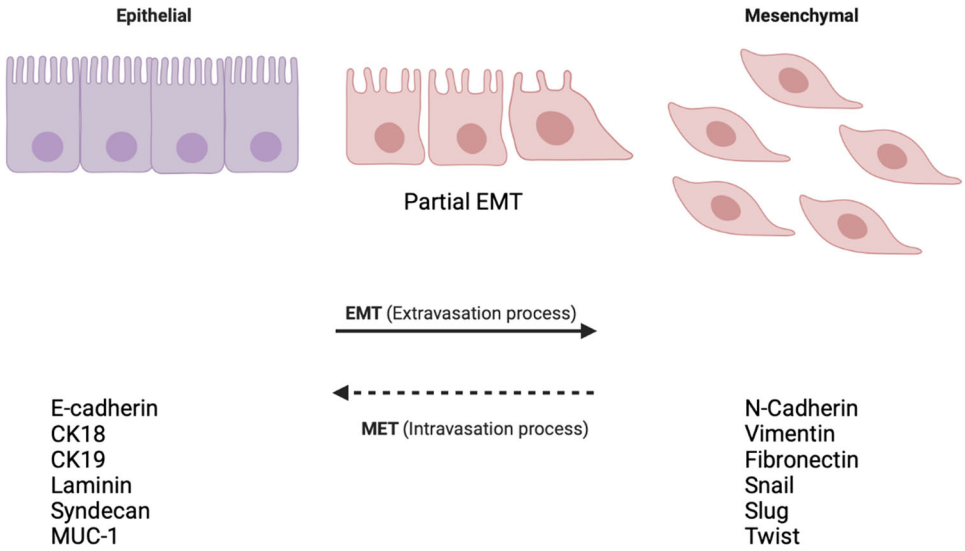

FIGURE 1. Epithelial cells lose their phenotype and gain a mesenchymal phenotype.

Fig. 1. During EMT, epithelial cells lose their epithelial phenotype, cell-cell adhesiveness, and gain a mesenchymal phenotype enabling them to migrate and invade.[68] An EMT process includes downregulating the expression of epithelial markers such as E-cadherin and cytokeratins, including CK18, CK19, laminin, Syndecan-1, MUC-1; and upregulating the expression of mesenchymal markers such as N-cadherin, vimentin, and fibronectin, Twist, Snail, and Slug.[79] In breast cancer progression, EMT is the early stage of invasiveness, as tumor cells often undergo the EMT program that facilitates cell dissemination.[120]

Signaling Pathways Responsible for Breast Cancer EMT

Regulators of EMT Pathways

EMT regulation involves various signaling pathways, including TGF-β, *Wnt*/β-catenin, Notch, TNF-α/ NF-κB, Hedgehog, and receptor tyrosine kinase (RTKs) signaling pathway.[39] These signaling pathways regulate transcription factors, such as Snail, Zeb1/ Zeb2, TWIST, miRNA, epigenetic regulators, and alternative splicing during breast cancer progression.[75] In addition to promote the transformation of early-stage primary tumors into invasive malignancies, EMT has been implicated in the generation of cancer cells with stem cell-like characteristics, including increased self-renewal, tumor-initiating capabilities, and resistance to apoptosis and chemotherapy.[109]

TGF-β Pathway in EMT

TGF-β is one of the crucial cytokines inducing EMT programming.[131] It plays a critical role in the activation of EMT and interacts with downstream signaling pathways during breast tumorigenesis.[45] It is an essential cytokine in cell growth and proliferation. Dysregulation in TGF-β expression leads to various carcinoma including breast carcinogenesis.[149] TGF-β signaling pathway is activated by intracellular Smad2/ 3 transducer proteins in EMT.[41] In Smad-dependent signaling, there are three forms of TGF-β: TGF-β1, TGF-β2, and TGF-β3 signaling associated with three different receptors: types I, II, and III. TGF-β binds to TGF-βR-II, leading to the activation of TGF-βR-I, which then induces the Smad2/3-dependent signaling pathways.[114] TGF-β receptors activate Smad2 and Smad3, leading to an activated complex of Smad2/3. Smad2/3 then complexes with Smad4.[136] This complex regulates several transcription factors that control the expression of many EMT related genes. This regulation includes direct phosphorylation of the receptors of SMAD transcription factors and cell polarity regulation proteins.[136] Studies suggested that a decrease in the expression of Smad2 and Smad3 links to the suppression of the invasiveness.[132] The upregulation of Smad2 and Smad3 expression, on the other hand, is linked to EMT. In non-Smad signaling pathways, TGF-β triggers the AKT/PI3K, Ras/Raf/MEK/ERK, and *Wnt*/β-catenin signaling pathways that induce the expression of epithelial proteins.[148] Both Smad-dependent and Non-Smad pathways work together to regulate the transcription factors, including Snail, Slug, ZEB1/2, and Twist.[60] TGF-β crosstalk with other signaling pathways, including Notch, *Wnt*/β-ca-

tenin, nuclear factor (NF)–κB, and RTKs, induces EMT and plays critical roles in maintaining the mesenchymal phenotype of invasive/metastatic tumor cells.[87] During EMT, TGF-β signaling alters the tight junction formation in epithelial cells and activates other signaling pathways, including *Wnt*, Notch, and Hh MAPK pathways. TGF-β regulates several gene expressions, including the transcription factor, SNAI1 and SNAI2/Slug, ZEB ZEB (ZEB1 and ZEB2/SIP1, Six family of homeobox (Six1), and Twist.[112] It then regulates transcription of E-cadherin, occludin and claudin.[87] In breast carcinoma, the upregulation of TGF-β is associated with an increase in EMT.[39] Recent study revealed its connection with breast cancer stem cells in EMT.

The Wnt/ β-catenin pathway in EMT

The *Wnt*/β-catenin pathway is considered exclusively crucial to the breast cancer EMT programming. A growing number of studies revealed that the *Wnt* signaling involves in the breast cancer cells' metastasis, immune microenvironment, stemness maintenance, and therapeutic resistance.[138] There has been 19 *Wnt* genes, 10 Frizzled (Fzd) receptors, and 2 low density lipoprotein receptor-related protein (LRP) co-receptors reported currently.[98]

The *Wnt* signaling is regulated by canonical or non-canonical signaling.[56] Canonical signaling is associated with β-catenin-dependent expression. It has been reported that β-catenin accumulation in nucleus is linked to a poor prognosis in breast cancer. On the other hand, non-canonical *Wnt* signaling is β-catenin-independent and do not lead to β-catenin expression in nucleus.[69] GSK-3β has been found to play a role in regulating β-catenin expression. It was reported that upregulation in GSK3β phosphorylation leads to the degradation of β-catenin, altering the *Wnt* signaling.[7] *Wnt* can stabilize the levels of Snail and β-catenin to induce EMT and cancer metastasis by blocking the activity of GSK-3β.[153]

Three *Wnt* signaling pathways, including *Wnt*/β-Catenin, *Wnt*–planar cell polarity (PCP), and *Wnt*–Ca_2 signaling, have been discovered for their roles in breast cancer progression. The *Wnt*/β-catenin is activated by the upregulation of SNAI1expression.[54] It leads to the downregulation of E-cadherin and upregulation of vimentin in breast cancer cells.[54] It has been reported that breast carcinoma subsets are induced by abnormal β-catenin expression and its subcellular localization, linked with the activation of the *Wnt* signaling pathway.[33] Some studies showed that the increase in β-catenin expression plays an important role in the development of breast carcinoma together with alterations in the *Wnt* pathway.[76] While the *Wnt* pathway is considered to be linked to the EMT in breast carcinoma, β-catenin alone is insufficient to induce EMT.[91] One of the studies in spindle lesions of the breast found the aberrant β-catenin expression in metaplastic breast carcinomas, suggesting the activation of *the Wnt* pathway in at least a subset of breast cancers.[58] β-catenin also acts as a molecular bridge to enhance cell-cell adhesion in the tight junctions of epithelial cells.[123] The stabilization of β-catenin and the activation of the *Wnt* signaling pathway are association with a transcription factor (TCF/LEF) and many other factors during EMT.[113]

Notch Pathway in EMT

In mammal cell development, the Notch pathway regulates cell survive. It plays a critical role in initiating and progressing cancer development.[65] Four Notch receptors (Notch1–4) and five ligands (Jagged1, 2 and Delta-like1, 3, 4) have been discovered.[125] The Notch signaling activates the NF-κB pathway and modulates TGF-β of the EMT programming.[24] Notch signaling is Numb-mediated. Numb is a negative regulator of EMT in both human epithelial cells and breast cancer cells. Reduced Numb expression is found to be associated with the upregulation of EMT.[147] Studies have revealed a potential connection between the overexpression of Notch signaling and the overall poor survival rate of breast cancer patients.[147] Notch signaling synchronizes with other pathways to induce EMT.[126] Notch signaling coordinates Jagged 1, a Notch ligand; and *HEY1*, a Notch target gene.[126]

Recent studies reported that the Notch signaling is linked to the maintenance of a cancer stem cell population in breast cancers.[144] The Notch signaling regulates the Snail expression either by transcriptionally activating Snail or *via* lysyl oxidase (LOX).[29] The Notch signaling upregulates LOX by activating a hypoxia-inducible factor 1-α (HIF-1α), which leads to stabilize Snail and ultimately results in upregulating EMT programming that causes the invasion of cancer cells.[102] It has been shown that hypoxia-induced Notch activation promotes EMT in breast cancer cells by upregulating the expression of HEY2 and HES1, resulting in an increase in cell migration and invasion of breast cancer.[110] Recent data suggested that breast carcinoma and increased Notch expression are linked to progression and poor prognosis.[57] Additional data implied that positive regulation of Slug by Jagged1-mediated activation of Notch IC leads to the suppression of E-Cadherin, causing EMT induction in breast cancers.[126] A number of studies suggested a link between hypoxia and Notch activity. Hypoxia is one of the critical regulators of tumor metastasis. Notch serves as a critical intermediate in conveying the hy-

poxic response into EMT.[47] Another study suggested an interplay between TGF-β and Notch activity. Increased Notch activity through Smad3 boosts Jagged1 and HEY1 expression, resulting in an increase in Slug expression and ultimately leading to the E-cadherin suppression.[126] Notch also regulates Snail-1 expression and lysyl oxidase (LOX).[102] The association of Snail with LOX expression and hypoxia signaling mechanisms has been speculated.[47]

TNF-α/ NF-κB signaling pathway in EMT

Tumor necrosis factor-α (TNF-α) is a 26 kDa transmembrane protein. It is cleaved into a soluble 17 kDa by TNF-α-converting enzyme (TACE).[44,134] It is an essential cytokine involved in several processes, including inflammation, cellular homeostasis, and tumor progression.[64] TNF-α promotes angiogenesis, invasion, and metastasis associated with the EMT reprogramming[134], through counteracting with E-cadherin and the activation of MMP9.[64] The induction of TNF-α in EMT is associated with the upregulation of Twist-1. The upregulation of TNF-α has shown to be associated with increased metastatic potential and invasiveness of breast cancer cells.[72] It has been shown that an increase in Twist-1 expression regulates EMT and cancer stemness properties induced by TNF-α.[152] Recent evidence suggested that Twist-1 expression promotes breast cancer cell metastasis in mice.[135] It has been discovered recently that a long-time exposure to TNFα activates IKKβ and NF-κB, resulting in the upregulation of the transcriptional repressor Twist1 and EMT as well as cancer stemness properties.[64,127] In particular, a study from breast cancer patients showed that there is an direct link between TNF-α production by peripheral blood T lymphocytes and circulating tumor cells expressing EMT markers.[63] Evidence suggested that the activation of NF-κB is associated with Snail, Slug, Twist, and ZEB1/ZEB2.[64] It was found that Snail can be stabilized *via* the activation of NF-κB by GSK-3β degradation in TNF-α/NF-κB activation pathway.[64] NF-κB is also responsible for the activation of vimentin and matrix metalloproteinases (MMPs) of mesenchymal cell markers.[100]

The hedgehog pathway in EMT

Another signaling pathway in breast cancer EMT is related to stem cell renewal hedgehog (Hh) pathway. The Hh pathway involves in embryonic development, stem cell renewal, and tissue homeostasis[4]. In the Hh pathway, three glioma-associated oncogene (GLI) transcription factors, including GLI1, GLI2, and GLI3, are responsible for activating or repressing the transcription of these factors.[80] A line of evidence suggested the contribution of the Hh pathways to EMT in breast cancers. Colavito, *et al*. reported that a high level of expression of GLI1 in breast cancer cells that undergo EMT.[15] It was also shown that the Hh pathway is associated with cancer stem cell properties and crosstalk between NFkB and GLI1.[105] As classified in the *Wnt* pathways, there are also canonical and non-canonical signaling in the Hh pathway. A study suggested that non-canonical activation of GLI1 *via* hypoxia or other inflammatory cytokines induces EMT, drug resistance, and invasion in breast cancer cells.[48] GLI1 expression and their contribution to EMT in breast cancers *via* the Hh pathway was confirmed using mouse models.[53]

Receptor tyrosine kinase (RTK) signaling pathway in EMT

There are several RTK identified for their contribution to EMT of breast cancer cells.[60] Hepatocyte growth factor (HGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) activate RTKs. HGF signals is associated with epithelial differentiation by downregulating E-cadherin and is linked to tumor metastasis. The HGF pathway is also associated with Snail transcription factor that induces EMT.[60]

MAPK or PI3K are two signaling that regulate EMT and invasion in breast cancer together with TGF-β.[39] Ras-activated MAPK induces EMT and invasion of breast cancer cells by promoting the Twist1 serine 68 phoshorylation and stabilization of PI3K signaling.[116] Studies also suggested that the *Wnt* and EGFR signaling are associated with the RTK pathways.[62] Although studies suggested the importance of the RTK signaling and their contribution to EMT, the EMT mechanism is composed of various signaling pathways. The activation of RTK itself may not be sufficient to elicit EMT. The contribution of other pathways, including TGF-β, *Wnt*, Notch, NF-κB, and ERK/MAPK pathways may lead to the EMT establishment.

Hypoxia in EMT

Hypoxia is a crucial factor in tumor microenvironment (TME) that regulates multiple hallmarks of cancer behaviors, such as angiogenesis, invasion, EMT, stemness and immune evasion.[77] Hypoxia is considered to be one of the major physiological causes for EMT.[60] It is associated with the HIF1α, hepatocyte growth factor (HGF), SNAI1, and TWIST1; the activation of the Notch or NF-κB pathways; and the induction of DNA hypomethylation.[60] In breast cancer development, the hypoxic microenvironment plays a prefunding role in the progression of the disease.[35] Hypoxia-related gene expression is linked to a poor

prognosis of the disease.[51] It has been reported that the expression of HIF-1α leads to endocrine therapy resistance to ERα$^+$ breast cancer cells.[51]

It has been shown that 3% oxygen levels can induce EMT, by inhibiting the activity of GSK3β and sparing β-catenin from phosphorylation and subsequent destruction.[61] In other studies, the activation of Notch signaling is required for hypoxia-induced EMT by activating the *Wnt*/β-catenin signaling with the resulting stimulation of Snail1.[126]

In addition to the HIF pathways, recent studies showed that there are also other signaling pathways in hypoxia that may be linked to EMT.[115] For example, AMP-activated protein kinase (AMPK), adenosine monophosphate (AMP)/adenosine triphosphate (ATP), or adenosine diphosphate (ADP)/ATP can also be upregulated by hypoxia.[115] It has been reported by Saxena, *et al*. that the AMPK activation could lead to the upregulation of TWIST1 expression that promotes EMT in breast cancer.[107] On contrary, Chou, *et al*. reported that the AMPK activation by another activator OSU-53 suppresses EMT induction by modulating the Akt-MDM2-Foxo3 signaling axis.[14] Another signaling pathway is PI3K-Akt-mTOR, a mediator that plays a big role in cell proliferation, nutrient uptake, anabolic reactions and autophagy.[40] This pathway is found to be activated by hypoxia.[40] PI3K-Akt-mTOR has been considered as a mediator of TGF-β signaling through non-SMAD pathway.[121] TGF-β may activate PI3K directly or by activating epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors in various cell types.[38] It has been found that PI3K-Akt-mTOR could influence HIF-1α activation, as mTOR is an upstream mediator of HIF-1α.[150] mTOR regulates its translation *via* phosphorylation of 4E-BP1, which in turn inhibits the interaction of eIF4E with translation initiation complex and results in mRNA translation activation that facilitates HIF-1α protein synthesis.[150] These controversial research results suggested that more studies are needed to prove hypoxia driven PI3K-Akt-mTOR in EMT.

Another pathway is the MAPK signaling. MAPK factors are hypoxia-related signaling. They are involved in EMT induction through non-SMAD TGF-β signaling.[115,83] There are three MAPKs, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MARK. Among them, ERK-mediated hypoxia inducts EMT through E-cadherin regulation.[20] Further studies suggested that type 1 and 3 ERK's along with other type of MAPKs including (JNK) and p38 MAPK are found to be able to stabilize the phosphorylation site of TWIST1 for EMT induction in breast cancer cells.[20] While hypoxia is always considered to be in a HIF-mediated manner, recent studies showed the potential inducement of EMT by AMPK, PI3K-Akt-mTOR and MAPKs, suggesting a non-HIF manner EMT induction. More studies are need to reveal the potential of mediation behind EMT.

MicroRNAs (MiRNA) in EMT

miRNAs are small noncoding RNAs that regulate gene expression by binding to and destroying a target mRNA, resulting in a reduction in protein expression encoded by the mRNAs.[108] For instance, miRNA-200c and MiR-9 have been found to regulate transcription factors, ZEB1 and ZEB2 and E-cadherin expression in non-small lung cancers to promote or suppress EMT.[81] These dual functions increase the complexity of revealing the miRNA's actual roles played in EMT programming. miRNA-200c is a member of the tumor suppressive family of miRNAs.[27] A line of evidence suggested that EMT is suppressed in triple negative breast cancer cells that do not express miRNA-200c.[89] Another miR-335 was found to be linked to the suppression of breast cancer.[143] A correlation between the expression of miR-335 and 20 primary breast tumors has been investigated extensively. It was reported that miR-335 downregulated metastatic genes in breast cancer, and the loss of miR-335 may serve as a negative prognostic indicator.[143] miR-300 was found to target to Twist and downregulate EMT and invasion of breast cancer.[151] The extensive review on the roles of miRNAs played in EMT programing of breast cancer can be found in the literature.[151,145]

The discovery of unique roles of miRNAs played in either suppressing or promoting breast cancer cells' EMT makes them a good target for drug development.[50] Nevertheless, the miRNA–mRNA interactions are complicated. Each miRNA may potentially target to hundreds of mRNAs, or vice versa. Since each small interfering RNA can interact with millions of target mRNAs, their impact on gene expression can be significant, however.[34] Therefore, more comprehensive analyses are necessary to understanding the miRNAs and their signaling pathways.[34,106]

Alternative Splicing and EMT

A line of evidence suggested that the alternation of mRNA splicing during EMT leads to the production of diverse protein isoforms in turned mesenchymal cells.[129] Such alternative splicing has been found in p120 catenin, CD44, the RTK, FGFR2 (REFS), etc. that regulate EMT.[129] Studies revealed that these alternative splicing are regulated by splicing regulatory proteins 1 (ESRP1) and 2 (ESRP2), two RNA proteins that control the splicing of many gene transcripts.[129]

The downregulation of these two proteins results in changes in mesenchymal protein isoforms, including adhesion, motility, and signaling pathways.[129] During EMT, other changes in splicing also occur, leading to an increase in EMT associated protein expression, including FOX1 homologue2 (RBFOX2), another RNA binding protein that promotes EMT and cell invasion.[3] There is another splicing factor, Ser-Arg-rich splicing factor 1 (SRSF1) that promotes EMT by splicing the mRNA encoding receptor of RTK, (RON1) and the GTPase RAC1.[37]

Snail /Slug/Twist

Slug, Snail, and Twist are transcription factors that regulate the expression of tumor suppression proteins.[71] Studies suggested that abnormal expression of these three transcription factors in breast cancer cells. It has been speculated that they play a key role in breast cancer progression.[140] Twist is indeed one of the transcription factors that play a role in cell migration and tissue development during embryogenesis.[140] It is also an important regulator in the EMT mechanism.[140] Twist expression is correlated to an increase in the expression of mesenchymal markers, such as fibronectin, vimentin, αSMA, and N-cadherin.[12] The upregulation of Twist expression is correlated to a poor prognosis in cancers.[93]

Snail and Slug are the members of Snail family sharing a SNAG domain at the N and C terminal regions that bind to E-boxes in the regulatory regions of target genes.[19] However, Snail-mediated histone modifications lead to the repressing of E-cadherin expression. The mechanism underlying these modification remains unknown.[86] Posttranscriptional modifications affect activity of the Snail1 and Snail2. These modifications involve many signaling factors, including TGFβ, Notch, tumor necrosis factor-α (TNF-α), EGF, FGF, *Wnt*, Shh, SCF/c-kit, hypoxia, and estrogens in breast cancer.[137]

Cancer Stem Cells (CSCs) and EMT

A growing evidence links EMT to CSCs, as more and more studies suggested that CSCs phenotype contributes to the metastasis and drug resistance.[94] Mani, *et al.* first detected presence of the stem cell $CD44^{high}CD24^{low}$ population in epithelial cells that undergo EMT.[70] More studies have shown that cells undergoing EMT can acquire stem cell-like characteristics.[111] However, the molecular connection between EMT and CSCs has not been explicated yet.[6] It has been suggested that breast epithelial cells undergoing EMT has similar gene expression profile as of mesenchymal stem cells.[133] Creighton, *et al.* demonstrated that breast cancers are found to be enriched in EMT and stem cell phenotype after endocrine (letrozole) and chemotherapy (docetaxel).[16] It is postulated that these cells may be tumor initiators and responsible for disease persistence, spreading, and refractoriness to chemotherapy.[16]

CSCs are able to undergo invasion, migration, survival, colonization, and metastasis.[67] In tumor development, CSCs are the ones that can self-renew and trigger tumorigenesis. They are $CD44^{+}/CD24^{-}$.[49] Breast cancer stem cells display undergoing EMT at the site of primary tumors. It has been understood that EMT-related genes in $CD44^{+}/CD24^{-}$ cells are expressed higher in primary breast cancer cells with stem cell features.[70]

CSCs are crucial to EMT. Their stem cell phenotype shows an increased resistance to apoptosis.[112] It has been reported that breast cancer patients undergoing chemotherapy/neoadjuvant therapy have a higher $CD44^{+}/CD24^{-}$ cell expressing EMT-associated genes that were found in the posttreatment biopsy.[103] Recent evidences suggested that the EMT may facilitate the generation of cancer cells with the mesenchymal features needed for dissemination and the self-renewal properties needed for initiating secondary tumors.[42] Another study suggested that the expression of Snail and Twist increases the tumor sphere formation in immortalized human mammary epithelial cells (HMLE) and yields more cells with a $CD44^{high}/CD24^{low}$ stem cell feature.[128]

Studies also suggested that there may be a direct link between EMT and CSCs-like properties, which may be prerequisites for cancer cell metastasis. A major driving force for these processes is the TGF-β signaling pathway.[112] Another signaling pathway, the ZEB/miR-200 is also associated with EMT-CSC linkage. This feedback loop of ZEB/miR-200 is a driving force for cancer progression towards metastasis by controlling the state of CSCs.[9] Recent evidences showed that the activation of β-catenin and the Akt pathways by Twist are critical for the maintenance of EMT-associated and CSC-like features.[66]

REMARKS

EMT mechanism manifests itself through various signal pathways, including TGF-β pathway, *Wnt*/β-catenin pathway, Notch pathway, and TNF-α/ NF-κB signaling pathway. These pathways orchestrate EMT and metastasis events in breast cancers. Each signal is a chain of cascades that affect one another. Examining these pathways leads to a hypothesis that the convergence of all these pathways on the transcription factors

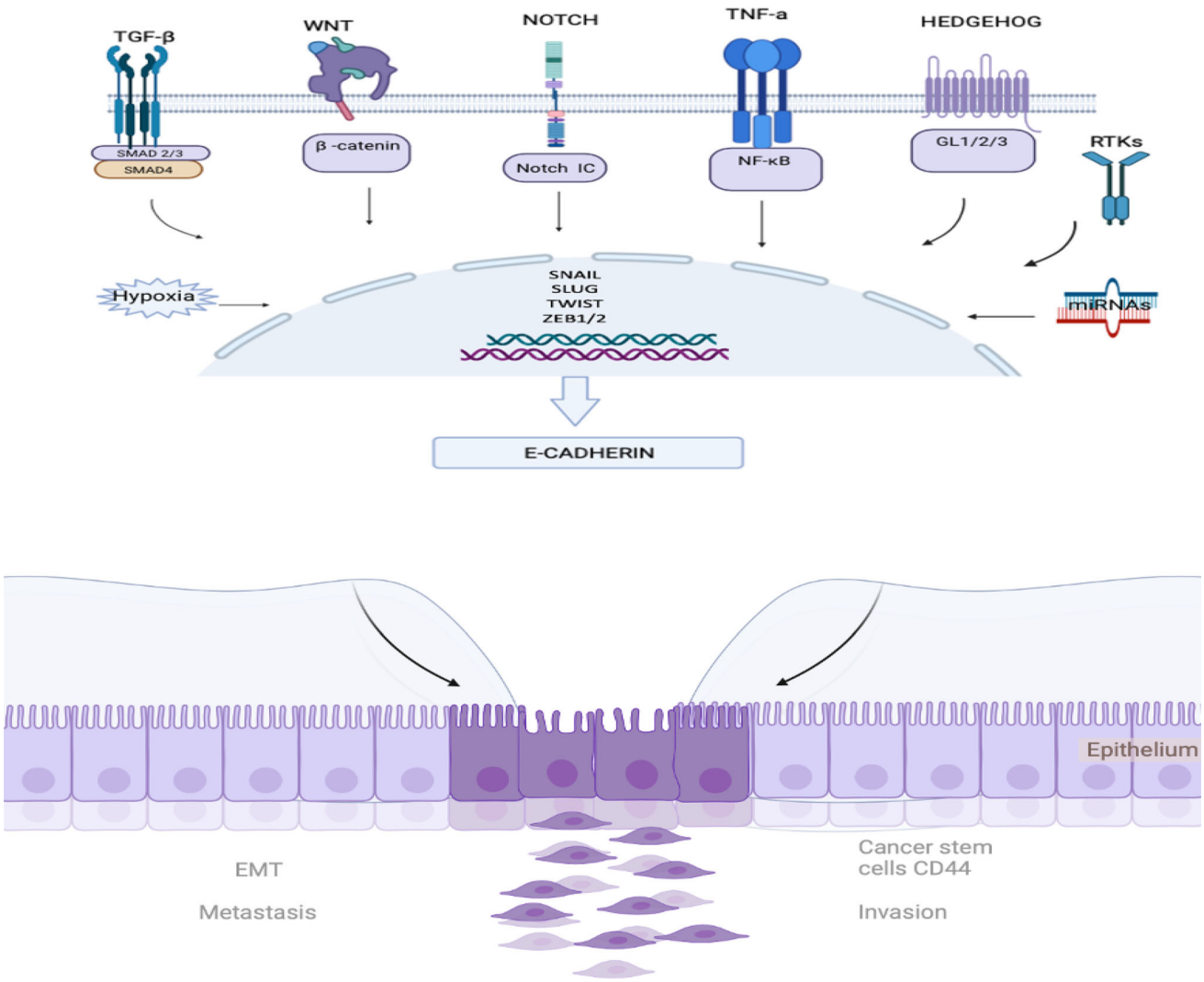

FIGURE 2. The convergence of EMT signaling pathways.

such as Snail, Zeb, and Twist leads to EMT, as illustrated in Fig. 2.

A growing evidence suggested that breast cancer stem cells affect EMT. Examining cancer stem cells in more detail revealed their relationship with the EMT and onset of the metastasis. Further study along these lines would shed light on the treating and/or preventing metastasis of breast cancer. In this context, understanding EMT mechanisms and determining external causes will help develop a better treatment for breast cancer cells.

CONFLICT OF INTEREST

B.B., S.J. and K.Y. have no conflicts of interest to disclose.

ETHICAL APPROVAL

No human subjects or animal research was performed as part of this study. The images were created in Biorender.com

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