Role of epithelial-mesenchymal transition in invasion and metastasis of breast cancers

J Liu1†, JX Shen1,2†, JL Hu1,2, XW Dou3, GJ Zhang2*.

Abstract

Introduction
Invasion and metastasis are the main causes for the death of patients with breast cancers. Epithelial–mesenchymal transition is implicated as a vital process in the invasion and metastasis of breast cancers, with endowing migratory and invasive cancer cells associated with metastatic capability. Increasing evidences demonstrated that epithelial–mesenchymal transition-initiating cells possessed mesenchymal features and stem-like traits that are resistant to chemotherapy. In this review, we summarise the physiological and pathological roles of epithelial–mesenchymal transition, new insights in the molecular mechanisms of regulating epithelial–mesenchymal transition during breast cancer invasion and metastasis, and its implication in chemotherapy resistance.

Conclusion
Therefore, it is challenging to probe and uncover the mechanistic regulation of oncogenic epithelial–mesenchymal transition, which will contribute to our understanding of metastatic dissemination and the role of targeting epithelial–mesenchymal transition with existing therapy as well as developing new drugs, in order to prevent metastasis and to diminish distant recurrence in patients with breast cancers.

Introduction
Breast cancer is the most common malignant tumour, and only second to lung and bronchus cancer as the cause of cancer-related deaths in women, which is often due to the development of metastatic disease1. For improving targeted treatments and accurate prognoses of patients with breast cancers, a comprehensive understanding of the mechanism of metastasis is an urgent requirement. Epithelial–mesenchymal transition (EMT), which is known as the probable first step in the complex process of metastasis, is a distinctive morphological change, in which the series of events converting the epithelial cancer cells switch from a well-differentiated, adherent phenotype to an individual, invasive migratory mesenchymal cell. EMT is vital for morphogenesis, such as physiological embryogenesis and histogenesis, and involved in multiple pathological processes, such as wound healing, renal fibrosis and tumour metastasis. EMT is mainly characterised by loss of E-cadherin (CDH1) expression and other special molecular changes that promote architectural changes, followed by the loss of cell–cell junction, cell–matrix adhesion and modulation of polarity, resulting in acquisition of mesenchymal features, such as spindle shape and increased migratory and invasive capacity. On the other hand, EMT is also supposed to be associated with cancer stem cells (CSCs), by generating self-renewing cells, which is contributed to the tumorigenesis and multi-drug resistance.

The aim of this review was to discuss the role of EMT in invasion and metastasis of breast cancers.

Discussion
Epithelial–mesenchymal transition and its classification
EMTs are involved in transdifferentiation of epithelial cells into mesenchymal cells under special physiological or pathological conditions. However, this process is reversible that the transdifferentiated migratory mesenchymal cells can re-generate epithelial cells, known as mesenchymal–epithelial transition (MET), or other cell types.

Based on different functional consequences, it is suggested to classify EMT into three biological subtypes. Type 1 EMTs, associated with implantation of a fertilised egg, embryogenesis and organ formation, can generate diverse cell types with mesenchymal phenotypes and potential to subsequently undergo a MET. Type 2 EMTs, associated with implantation of a fertilised egg, embryogenesis and organ formation, can generate diverse cell types with mesenchymal phenotypes and potential to subsequently undergo a MET. Type 3 EMTs, associated with implantation of a fertilised egg, embryogenesis and organ formation, can generate diverse cell types with mesenchymal phenotypes and potential to subsequently undergo a MET.

Type 1 EMTs, associated with implantation of a fertilised egg, embryogenesis and organ formation, can generate diverse cell types with mesenchymal phenotypes and potential to subsequently undergo a MET. Type 2 EMTs, associated with implantation of a fertilised egg, embryogenesis and organ formation, can generate diverse cell types with mesenchymal phenotypes and potential to subsequently undergo a MET. Type 3 EMTs, associated with implantation of a fertilised egg, embryogenesis and organ formation, can generate diverse cell types with mesenchymal phenotypes and potential to subsequently undergo a MET.
Type 3 EMTs occur in epithelial cancer cells, with genetic and epigenetic differences from normal epithelial cells, specifically in genes that favour clonal outgrowth and the development process of localised tumours. The epithelial cancer cells undergo a type 3 EMT and convert to a mesenchymal phenotype, resulting in invasive and metastatic characteristics, and subsequently generate the high malignant, potentially lethal behaviour of cancer progression. Till date, it is still unclear what specific signals trigger type 3 EMT in carcinoma, how and when EMT and MET programmes and the genes involved in these processes are coordinated (Figure 1).

**Breast cancer epithelial–mesenchymal transition regulators and signalling pathways**

A hallmark of EMT is losing expression of E-cadherin, which is the key cell–cell adhesion molecule and caretaker of the epithelial phenotype. Besides E-cadherin, many other markers are used to distinguish between epithelial and mesenchymal-like phenotypes, such as downregulation of plakoglobin, occluding, α-catenin, claudins 3/4/7, and upregulation of vimentin, fibronectin, ACTA2. Importantly, carcinoma cells may undergo EMTs to different extents and not completely lose their epithelial phenotype during the process, with some cells expressing epithelial and mesenchymal markers simultaneously and other cells becoming completely mesenchymal.

**Transcription regulation**

It is reported that loss of CDH1 is sufficient to induce EMTs, and sequent invasive and anoikis resistance, indicating the significance of repressors of CDH1 in the trigger of EMTs. A number of transcription factors are EMT inducers by direct transcriptional repression of CDH1, such as the basic helix–loop–helix factors (E12/E47 and Twist), the zinc-finger proteins (Snail, and Slug) and the ZEB family (ZEB1 and SIP1) that recognise the E-Box DNA sequence located near the transcriptional initiation site of CDH1, recruiting transcriptional co-factors and histone deacetylases.

Snail was the first discovered and expressed in all EMT processes, which can be induced through signalling involved in EMT, including tyrosine and serine/threonine kinase receptor-associated pathways, and Wnt signalling pathways. Besides repression of E-cadherin, Snail also downregulates the expression of other epithelial molecules, and induces the expression of genes associated with a mesenchymal and migratory phenotype. Recent research shows that Snail protects the breast cancer cell from hypoxia attack, at least partly via beta-catenin which upregulated expression of HIF-1 dependent genes. Snail interacted with Suv39H1, a major methyltransferase responsible for H3K9me3 that intimately links to DNA methylation, and recruited it to the promoter of E-cadherin for transcriptional repression, and knockdown of Suv39H1 restored E-cadherin expression by blocking H3K9me3 and DNA methylation and...
resulted in the inhibition of cell migration, invasion and metastasis of basal-like breast cancer.11

Twist, regulated by Wnt/beta-catenin signalling, is highly expressed in breast cancer tissues, playing important roles in mammary tumourigenesis, with the reverse E-cadherin expression.12 Stable overexpression of Twist in the breast cancer cell line, MCF-7, induced its morphology to a fibroblastic-like phenotype, which exhibited protein markers representative of a mesenchymal transformation, and resulted in angiogenesis through upregulating vascular endothelial growth factor.13 An autocrine MAPK intracellular signalling pathway, activated by OPN extracellular binding to MB231, resulted in Twist activation and promoted Bmi1 expression, and further EMT initiation and cellular migration.14

microRNA

microRNAs (miRNAs) play an important role in development as well as in the induction of EMT by targeting and directing cleavage of the special miRNAs, or translational inhibition. miR-200 family are the most frequently cited EMT-related miRNAs, including miR-200a/b/c, miR-141 and miR-429, through downregulating the E-cadherin transcriptional repressors, ZEB1/2, at least partially. On the other hand, ZEB1/2 can repress transcription of miR-200s directly, constructing a reciprocal repression between ZEB and miR-200s.15

Recently, other targets of miR-200s have been reported to miR-200s regulates tumour cell plasticity and metastasis through a moesin-dependent pathway, distinct from the ZEB1/E-cadherin axis.16

Besides E-cadherin, miRNAs can also target other EMT-associated molecules and influence the EMT processes. miR-21s, one of the first mammalian miRNAs identified, have multiple tumour suppressor targets, such as PTEN, which can be upregulated in MDA-MB-231 breast cancer cells, accompanied with AKT/ERK1/2 inactivation and reversing the EMT and CSC phenotype.17 It is reported that re-expression of miR-375, targeting metastadherin, was sufficient to sensitise tamoxifen-resistant breast cancer cells to tamoxifen and partly reversed EMT.18 Six1 activates the tumour promotional arm of transforming growth factor-β (TGF-β) signalling, via upregulating the miR-106b-25 miRNA cluster; and the miR-106b-25 cluster is sufficient to induce an EMT and a tumour-initiating cell phenotype.19

Transforming growth factor-β signalling pathway

TGF-β, a cytokine produced by mesenchymal stromal and inflammatory cells, regulates cell proliferation, cell differentiation, apoptosis and matrix accumulation, and is probably implicated as the primary inducer of EMT. It is now generally accepted that TGF-β signalling plays a dual role in tumour carcinogenesis and metastasis. Although during the early stages of carcinogenesis, TGF-β acts as a tumour suppressor, via upregulating cyclin-dependent kinase (CDK) inhibitors (p15 and p21) and shut-down expression of their repressor Myc.20 It promotes metastasis through facilitating induction of EMT at advanced stages of carcinogenesis.21 TGF-β, binding to type I/II serine-threonine kinase receptors, regulates transcription by Smad-dependent/independent TGF-β-receptor signalling pathways. And both Smad-dependent/independent pathways overlap and collaborate to regulate the transcription of EMT regulators, including Snail, Slug and Twist.22

TGF-β signalling downregulates expression of E-cadherin, via Snail1/2, HMGA2 and ZEB1/2. CDK5 is commonly overexpressed and significantly correlated with several poor prognostic parameters of breast cancer, and essential for TGF-β1-induced EMT and breast cancer progression.23

Pyk2 expression was inversely related to that of E-cadherin, and Pyk2 deficiency prevented TGF-β from stimulating the growth of breast cancer cells, indicating that Pyk2 mediates distinct elements of the EMT programme and metastatic cascade regulated by TGF-β, particularly the initiation of secondary tumour outgrowth by disseminated cells.24 In different subtypes of breast cancer, the signalling pathways involved in TGF-β-mediated EMT are not exactly the same. Recently, research showed that upregulated WAVE3 expression is essential for TGF-β-mediated EMT and metastasis of triple-negative breast cancer cells.25 Significant cross-talk exists between TGF-β and other signalling pathways, such as Notch, Wnt, NF-kB and RTKs, to induce EMT and promote invasive phenotype of breast cancer cells, such as Six1, which can activate both TGF-β and Wnt signalling.

Notch and Wnt signalling pathways

Notch and Wnt signalling pathways play essential roles in development and maintenance of organs and tissues, involved in cell proliferation, survival, apoptosis and differentiation, as well as stem cell self-renewal. And it is believed that Notch and Wnt signalling pathways may play an important role in the induction of EMT and the sequent progression of breast carcinomas.

Mammals express four transmembrane Notch receptors (Notch1/2/3/4) and five canonical transmembrane ligands (Jagged-1/2 and Delta-like-1/3/4). Cell–cell contact is generally necessary for activation of Notch signalling, through ligand–receptor binding and proteolytic cleavage sequence and release of Notch intracellular domain, which translocates to the nucleus to activate Notch target genes, including Myc, p21, HES and HES-related repressor families (HERP, HRT and HEY).26 Slug was reported to be essential for Notch-mediated repression of E-cadherin.
resulting in β-catenin activation and EMT in breast cancer cells\textsuperscript{27}. TGF-β increases Notch activity through Smad3, which upregulates both Jagged-1 and Hey-1, and subsequently promotes Slug expression, resulting in suppressing E-cadherin\textsuperscript{27}. And TGF-β-induced EMT can be blocked by knockdown of Hey-1 or Jagged-1 or by pharmacological inactivation of Notch\textsuperscript{28}. Bone is hypoxic and one of the most frequently targeted organs for breast cancer metastasis, and Jagged-2 is upregulated in bone marrow stroma under hypoxia, which significantly promotes EMT and self-renewal of breast CSCs\textsuperscript{29}, indicating a vital role of Notch-induced EMT in tumour progression and metastasis. And hypoxia-induced downregulation of miR-34a can promote EMT by directly targeting the Notch1 and Jagged-1, and subsequently, Notch downstream signalling in tubular epithelial cells\textsuperscript{30}.

β-catenin, one of the downstream signalling molecules of the Wnt signalling pathway, can enhance cell–cell adhesion when bound to cadherin complexes in adherens junctions. In the absence of Wnt, cytoplasmic β-catenin is phosphorylated by the destruction complex, consisting of axin, adenomatous polyposis coli, glycogen synthase kinase-3β (GSK-3β) and casein kinase, recognised by E3 ubiquitin ligase β-Trcp for proteasomal degradation. In the presence of Wnt ligands, GSK-3β is inactivated, and β-catenin is stabilised, resulting in translocation into the nucleus and regulates the transcription of Wnt target genes, such as Snail and Slug\textsuperscript{31}. Snail can functionally interact with β-catenin to enhance the activation of Wnt signalling, which establishes a positive feedback loop to Wnt-dependent transcription. It is reported that repression of Wnt/β-catenin signalling can prevent EMT and sequent metastasis of basal-like breast cancer to the lungs\textsuperscript{32}. In trastuzumab-resistant HER2-overexpressing breast cancer cells, expression of Wnt3 activates Wnt/β-catenin pathway that leads to transcription of EGF4 and promotes EMT-like transition\textsuperscript{33}.

**Roles of epithelial–mesenchymal transitions in cancer stem cells and chemotherapy resistance**

As both EMT and CSC phenotypes are implicated in metastasis, it is proposed and demonstrated as a connection between them. A recent study demonstrated that mitochondrial DNA retrograde generates EMT and CSCs in human mammary epithelial cells (HMECs)\textsuperscript{34}. Recent research studies indicated that the induction of EMT by ectopic expression of Snail, Twist or treatment with TGF-β in immortalised HMECs yields cells with CD44\textsuperscript{high}/CD24\textsuperscript{low} stem cell phenotype, acquiring the ability to form tumour spheres, suggesting that the EMT process may facilitate the generation of cancer cells with the mesenchymal traits for dissemination as well as CSC properties for self-renewal and initiating secondary tumours\textsuperscript{35}. In addition, CD44\textsuperscript{high}/CD24\textsuperscript{low} population of normal and transformed HMECs displayed EMT-associated phenotypes, compared with CD44\textsuperscript{low}/CD24\textsuperscript{high} cells\textsuperscript{36}. Isolated CD44\textsuperscript{high}/CD24\textsuperscript{low} stem-like cells from normal and neoplastic human mammary cells exhibited a mesenchymal morphology and expressed mesenchymal markers, such vimentin and fibronectin\textsuperscript{35}.

Twist directly stimulates the expression of Bmi1 to maintain self-renewal and represses expression of E-cadherin and p16INK4a, with cooperation of Bmi1, thus simultaneously promoting EMT and conferring tumour-initiating capabilities of CSCs\textsuperscript{37}. Twist also modulates the CSC phenotype through downregulating the expression of CD24\textsuperscript{38}. Besides Twist, Six1, Snail and TGF-β can induce an EMT and increase breast CSC features. Interestingly, miRNAs has added the complexity to the networks regulating EMT and CSC, miR-200s also target Bmi1, which is a positive regulator in self-renewal of CSCs and breast cancer metastases\textsuperscript{40}.

Interestingly, the EMT-resulting mesenchymal tumour cell had a CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype with the ability to re-establish an epithelial tumour and increased drug resistance, consistent with breast CSCs signatures\textsuperscript{41}. Many patients with breast cancers ultimately relapse due to the presence of residual cancer cells that are presumably treatment resistant. Examination of breast tumours after neo-adjuvant chemotherapy revealed an increase in the CSC-enriched CD44\textsuperscript{high}/CD24\textsuperscript{low} population, importantly, samples obtained after treatment were also enriched in EMT-related mesenchymal markers, highlighting the relationship between oncogenic EMT and CSCs\textsuperscript{42}. Several colony clusters from SKBR3 cell lines undergoing a spontaneous EMT in vitro, expressed conventional EMT markers and a predominant CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype with decreased HER2 expression, suggesting the mechanisms of trastuzumab resistance in HER2-positive breast cancers\textsuperscript{43}. Acquisition of the EMT phenotype in various derivatives of MCF-7 human breast cancer cells was associated with CD44\textsuperscript{high}/CD24\textsuperscript{low}/ALDH\textsuperscript{high} stem cell populations, as well as with an inhibition of CTL-mediated tumour cell lysis strikingly, resulting in escaping from T-cell-mediated immune lysis\textsuperscript{44}.

**Conclusion**

Breast carcinoma is a heterogeneous group of neoplasms, with a high degree of diversity of gene expression profiling between and within tumours, representing a variety of
histopathological features, genetic markers, risk of disease progression, prognostic outcomes and therapeutic resistance. Conventional treatment for breast cancers mainly targets the differentiated tumour cells; however, cancer cells in a significant amount of patients can acquire chemotherapy resistance after standard therapies, resulting in relapse or metastasis with poor prognosis. The link between EMT and CSCs is widely accepted that a minor population of tumour cells, with EMT and CSCs traits, may respond for tumour metastasis and recurrence. Therefore, it is challenging to probe and uncover the mechanistic regulation of oncogetic EMT, which will contribute to our understanding of metastatic dissemination and the role of targeting EMT with existing therapy as well as developing new drugs, in order to prevent metastasis and to diminish distant recurrence in patients with breast cancers.

Abbreviations list
CDK, cyclin-dependent kinase; CSC, cancer stem cell; MET, mesenchymal–epithelial transition; EMT, epithelial–mesenchymal transition; GSK-3β, glycogen synthase kinase-3β; HMEC, human mammary epithelial cells; miRNAs, microRNAs; TGF-β, transforming growth factor-β.

Acknowledgment
This study was supported by the State Key Development Program for Basic Research of China (No. 2011CB707705), the National Natural Science Foundation of China (No. 31271068 and 81320108015) and the Seed funding of Guangdong Provincial Key Laboratory for Diagnosis and Treatment of Breast Cancer.

References
26. Grego-Bessa J, Diez J, Timmerman L, de la Pompa JL. Notch and epithelial-mesenchymal transition in development and competition for nutrients, cancer-derived inflammatory cytokines, and other factors that modulate cell survival and proliferation.