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TOPIC HIGHLIGHT

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Epithelial-mesenchymal transition transcription factors and miRNAs: "Plastic surgeons" of breast cancer

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Abstract

Growing evidence suggests that breast cancer cell plasticity arises due to a partial reactivation of epithelial-mesenchymal transition (EMT) programs in order to give cells pluripotency, leading to a stemness-like phenotype. A complete EMT would be a dead end program that would render cells unable to fully metastasize to distant organs. Evoking the EMT-mesenchymal-to-epithelial transition (MET) cascade promotes successful colonization of distal target tissues. It is unlikely that direct reprogramming or trans-differentiation without passing through a pluripotent stage would be the preferred mechanism during tumor progression. This review focuses on key EMT transcriptional regulators, EMT-transcription factors involved in EMT (TFs) and the miRNA pathway, which are deregulated in breast cancer, and discusses their implications in cancer cell plasticity. Cross-regulation between EMT-TFs and miRNAs, where miRNAs act as co-repressors or co-activators, appears to be a pivotal mechanism for breast cancer cells to acquire a stem cell-like state, which is implicated both in breast metastases and tumor recurrence. As a master regulator of miRNA biogenesis, the ribonuclease type Ⅲ endonuclease Dicer plays a central role in EMT-TFs/miRNAs regulating networks. All these EMT-MET key regulators represent valuable new prognostic and predictive markers for breast cancer as well as promising new targets for drug-resistant breast cancers.

Key words: Embryonic transcription factors; Epithelial to mesenchymal transition; Breast cancer; MicroRNAs; Dicer; Feedback loop

Core tip: Epithelial-mesenchymal transition (EMT) and the reverse mesenchymal-epithelial transition (MET) are both involved in breast cancer plasticity. Embryonic transcription factors and miRNAs are key players regulating the balance between these two processes allowing cells that underwent EMT to transiently re-acquire epithelial phenotype. Here we highlighted the complex transcription factors/miRNAs regulation networks involved in EMT-MET during breast cancer progression and the central role played by Dicer, the key enzyme of miRNAs processing, in EMT process. These key regulators of EMT-MET may represent predictive markers and potential therapeutic targets for breast cancers.

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EMT AND MET REPROGRAMMING DURING BREAST CANCER PROGRESSION

During embryonic development, a complex organism is formed from a single starting cell. Growth and differentiation are driven by large transcriptional changes, directed by the expression and activity of transcription factors (TFs). Cancer is often suggested to imperfectly resemble the development process by re-expressing certain embryonic TFs. Links between normal embryonic development and cancer biology have been postulated, but no defined genetic/epigenetic basis has been established. During normal development, cells divide, align themselves, and specialize to form discrete tissues and organs. For the body to develop properly, cells must coordinate their migratory patterns and the process by which they differentiate or evolve from less-specialized cells into more-specialized cell types. A lack of such coordination leads to disordered development and, in some cases, cancer.

The mammary gland is an organ that undergoes distinct and complex developmental stages after birth. Post-natally, mammary ducts elongate into the mammary fat pad. Terminal end buds, the highly proliferative structures found at the tips of the invading ducts, expand and increase greatly after birth. By puberty, the mammary ducts have invaded to the end of the mammary fat pad. At this point, the terminal end buds become less proliferative and decrease in size. Side branches form from the primary ducts and begin to fill the mammary fat pad. Ductal development decreases with the arrival of sexual maturity and undergoes estrous cycles. As a result of estrous cycling, the mammary gland undergoes dynamic changes where cells proliferate and then regress. During each estrus cycle, the density of ductal branches and alveolar buds increases. During pregnancy, the alveolar buds formed on the ductal tree give rise to large, lobulo-alveolar differentiated structures capable of milk production. Understanding how the mammary tissue develops and functions is of great importance in determining how its control mechanisms break down in breast cancer. The leucine-rich repeat containing G protein-coupled receptor 4 (Lgr4) has been implicated in mammary development and stem cell activity, with *Lgr4*-/- mice showing delayed ductal development, fewer terminal end buds, and decreased side-branching mediated by the Wnt/β-catenin/Lef1 pathway and Sox2[1]. An article from the Breakthrough Breast Cancer Research Centre has recently compared an embryonic mammary epithelial signature with *Brca1*-deficient mouse mammary tumors and human breast cancer signatures. Specific subsets of embryonic mammary genes were found over-expressed both in mouse *Brca1*-/- tumors and in human basal-like cancers[2]. Reactivation of a small network of embryonic mammary programs within differentiated tumor cells may elicit cell behavior associated with a stem-like, highly plastic state. The EMT-mesenchymal-to-epithelial transition (MET) cascade, although an intrinsic part of normal developmental processes during organogenesis, is also recognized as a critical event for metastasis of carcinomas[3,4]. EMT allows tumor cells to de-differentiate and to acquire motility and invasive properties in order to spread into distant organs, and the MET program then reboots an epithelial program to establish new tumors at the sites of dissemination[5]. It is not entirely known how and when EMT and MET programs, and the genes associated with these processes, are coordinated. The hallmark of EMT is the loss of adherent junctions through loss of E-cadherin (CDH1) expression. E-cadherin repressors fall into two groups, direct or indirect regulators, depending on whether or not they bind the *CDH1* promoter.

EMT INDUCERS

The powerful direct repressors of *CDH1*, playing a major role in EMT, originate from three distinct families: The Snail family comprises three members, Snai1, 2 and 3 (also termed Snail, Slug and Smuc), the Zeb family (Zeb1/2), which are zinc-finger transcription factors that recognize a consensus E-box type element, and the b-HLH family (TWIST1/2) which also bind to a consensus E-Box sequence, as homo- or heterodimers. These factors also repress the transcription of several other junctional proteins, including claudins and desmosomes. The other group of *CDH1* repressors (indirect regulators) comprises FoxC2, Goosecoid, TCF4, paired mesoderm homeobox protein 1 (PRRX1), and some Sox family members. FoxC2 is a winged helix/Forkhead domain transcription factor, which lies downstream of Twist, Snail and Goosecoid, and affects E-cadherin expression by promoting its cytoplasmic localization. They increase the invasiveness of epithelial cells (Table 1). The third member of the Snail family, (Smuc) does not play a major role during EMT, while Zeb1 is a strong motility driver. The Snail, Zeb and TWIST families operate within a complex regulatory network where they activate or repress each other. EMT inducers, such as EMT-TFs (TWIST1, Snail, Slug, and Zeb1), can also confer 'stemness' as demonstrated in several studies, where the induction of EMT enhances self-renewal and the acquisition of CSC (Cancer Stem Cell) characteristics[6-8] (Table 1). In contrast, several studies show that tumor cells with an epithelial phenotype survive in the circulation and form distant metastases[9-12]. It has been demonstrated that the mesenchymal phenotype does not facilitate metastatic progression; rather, most cancers invade and travel through lymphatic and blood vessels *via* cohesive epithelial migration, and do not undergo EMT-MET[13]. Interestingly, Zvelebil *et al*[2] showed that enrichment for the mammary mesenchymal gene signature (TGFβI, TWIST2, ZEB2) was correlated with large tumor size, but no significant association with

Table 1 Involvement of epithelial-mesenchymal transition-transcription factors in breast carcinogenesis

EMT-TF	Transcription factor type	Deregulated in breast cancer	Association with biological and clinico-pathological features in breast cancer
SNAI1 (Snail)	Zinc finger	High levels[75]	Lymph node metastasis, effusion, distant metastasis, recurrence
SNAI2 (Slug)	Zinc finger	High levels[76]	Effusion, distant metastasis, recurrence, stemness capacities
TWIST1	Basic Helix-loop-Helix	Up-regulated[77]	Primary transformation, escape from failsafe programs, invasion, bone metastasis, angiogenesis, poor prognosis
ZEB1	Zinc finger E-box-binding homeobox 1	High levels[76,77]	Invasion, distant metastasis
ZEB2	Zinc finger E-box-binding homeobox 2	High levels[76,77]	Invasion, distant metastasis, stemness capacities
FoxC2	Forkhead-related protein FKHL14, FKH-14, mesenchyme fork head protein 1	High levels[78]	Stemness capacities, distant metastasis
Oct3/4	Octamer-binding transcription factor 4, POU domain, class 5, transcription factor 1S homeodomain transcription factor of the POU family	Up-regulated[79]	Stemness capacities, invasion, migration
Sox2	Sex determining region Y-box 2 highly conserved DNA binding domains High-mobility group box domains	Up-regulated[80]	Tamoxifen-resistance, lymph node metastasis, stemness capacities
Prrx1	Paired related homeobox 1	High levels[15]	Metastasis, poor prognosis
TCF4	Basic Helix-loop-Helix immunoglobulin transcription factor 2	Up-regulated[81,82]	Metastasis, poorer prognosis in patients with high levels of osteopontin and better prognosis with low levels of osteopontin

EMT-TF: Epithelial-mesenchymal transition-transcription factors.

overall survival was observed in patients whose breast cancers showed activation of the embryonic mesenchymal signature. Four transcription factors (Bcl11a, Grhl3, Prox1, Sox11) activated in *Brca1-/-* mouse tumors and basal-like human breast cancers were confirmed to be embryonic-enriched and highly expressed by some tumors. Increasingly, evidence points towards the transient involvement of an activated EMT program in the invasive front of tumors rather than in dissemination of cancer cells. The mesenchymal transcriptomic program is found associated with metaplastic breast carcinoma (MBC), a rare tumor with a carcinosarcoma-like aspect, with a larger tumor size, accounting for < 1% of all breast cancers. Histologic subtypes identified were chondroid (24%), spindle (20%), sarcomatoid (16%), squamous (11%) and mixed (29%), with the origin of the "mixed" subtype hypothesized to be from the "differentiation" of immature breast glandular epithelial cells into non-glandular mesenchymal tissue. In prostate cancer cell lines, subpopulations with a strong epithelial gene program were enriched in highly metastatic tumor-initiating cells (TICs), whereas mesenchymal subpopulations showed reduced TIC[12]. Are epithelial cancer cells expressing EMT-TFs without experiencing a full EMT program the most prominent to metastasize? The answer is no for some factors, as it was recently shown that TWIST1 down-regulation and a subsequent re-differentiation (MET) at the distant site is necessary to allow colonization and macrometastasis[14]. Intriguingly, the EMT-inducer Prrx1 suppresses stemness traits[15] and a knockdown of Prrx1 and TWIST1 increased lung metastasis after tail vein injection.

The question of whether the MET is stable in the metastases or if these cells show ongoing phenotypic plasticity leading to a second EMT is also an open question. Collectively, these results illustrate the plasticity governing self-renewal and mesenchymal gene interactions (Figure 1).

INVOLVEMENT OF DICER IN EMT

Genes central to gene regulatory networks (GRNs) may have a huge impact on cell plasticity. The ribonuclease type Ⅲ endonuclease Dicer, involved in the RNA interference process, belongs to this gene category. RNA interference (RNAi) and microRNA (miRNA) pathways are conserved, post-transcriptional gene silencing mechanisms in which single-stranded guide RNAs bind to cognate mRNAs and direct their endonucleolytic cleavage or translational repression by RNA-induced silencing complexes (RISCs). An important function of Dicer is to process miRNA precursors into approximately 22-nucleotide non-coding small RNAs. As a master regulator of miRNA biogenesis, Dicer is involved in EMT, cancer cell plasticity and tumor progression. We have found that Dicer mRNA expression was variable in breast carcinoma samples and that lower levels were more frequent in patients with metastatic relapse, indicating that Dicer mRNA levels are clinically relevant as reported by Grelier *et al*[16]. In accordance with other studies, we have found a global decrease of miRNA expression in correlation with the decrease of Dicer expression[17]. Levels of Dicer are tightly controlled to maintain the homeostasis of miRNA production, largely at the post-transcriptional level. Dicer is a highly conserved protein that is found in almost all eukaryotic organisms. Some organisms contain multiple *Dicer* homologues, whereby different Dicer isotypes have distinct roles, for instance *D. melanogaster* Dicer-1 is required for miRNA biogenesis, whereas Dicer-2 functions in siRNA production. Contrary to other organisms, mammals have a single *Dicer* gene, but its expression is a highly regulated process with spliced Dicer mRNAs putatively encoding both spliced and full-length pro-

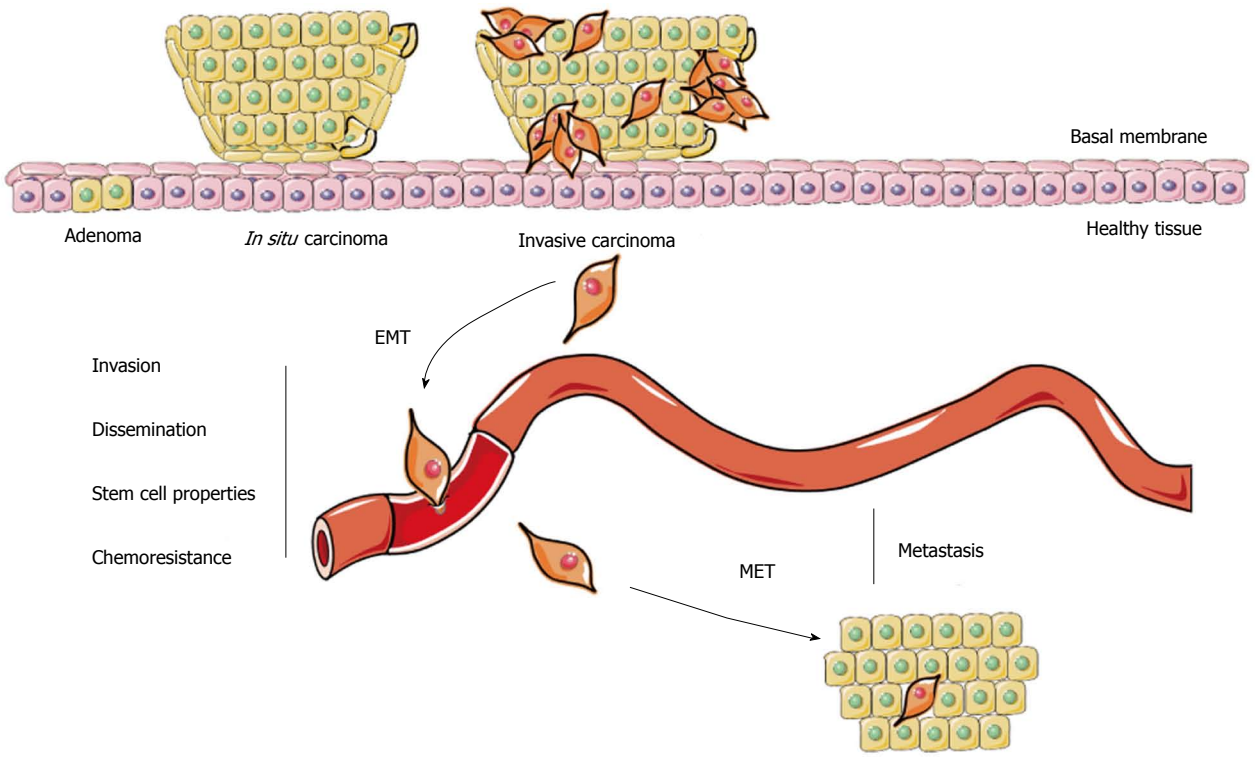

Figure 1 Epithelial to mesenchymal transition and mesenchymal to epithelial transition during breast tumor progression. During tumor progression, cancer cells undergo apithelial to mesenchymal transition (EMT) to acquire invasive, dissemination, chemoresistance and stem cell properties. Thus, an *in situ* carcinoma progresses to an invasive carcinoma and cells disseminate throughout the entire body *via* the blood and the lymphatic vessels. After dissemination, cells must undergo an mesenchymal to epithelial transition (MET) to colonize distant organs.

teins. In humans, there are 3 full-length isoforms showing considerable differences in their 3'UTR sequence. Only two variants exhibit a long 3'UTR sequence, while the third variant exhibits a very short 3'UTR lacking all predicted miRNA target sites[18,19]. Moreover, we identified two splice variants which were highly expressed in some breast cancer cell lines, yet totally absent in others. Theoretically, these isoforms may be functional as they both contain the ribonuclease Ⅲ domain and the dsRNA binding domain, while one isoform contains only a PAZ domain[18]. We have shown that the full-length Dicer protein decreased during the EMT process[16]. The presence of spliced forms was correlated with epithelial/mesenchymal phenotype. Indeed, in almost all cell lines that exhibit a complete or partial mesenchymal phenotype, these truncated isoforms were not detectable by western blot as shown by Hinkal *et al*[18]. Conversely, epithelial cells expressed easily-detectable levels of the two variants. Furthermore, we have found decreased expression of these variants during EMT using immortalized human epithelial mammary cells transfected by RAS. These data imply an integral role for internal site miRNA regulation of Dicer isoforms, but the physiological relevance of these data remains to be clarified. Thomas Duchaine's group has shown the presence of a truncated form of Dicer in *C. elegans*, corresponding to a C-terminal fragment (sDCR-1). They demonstrated that sDCR-1 operates independently of full-length Dicer in two distinct RNAi pathways; it enhances exogenous RNAi (exoRNAi) and concurrently acts as a negative regulator of microRNA (miRNA) biogenesis[20]. Interestingly, one of the spliced form variants we identified in epithelial breast cancer cell lines encodes a protein sharing the same domains as sDCR-1. By ectopically expressing this isoform in HEK293T cells, they have found that, similar to the function of sDCR-1, there was a decrease in accumulation of mature-to-precursor forms for some miRNA but not all, showing that this function is miRNA-specific[20]. Deciphering the role of the highly conserved Dicer variants is of great importance since Dicer acts as a tumor suppressor in specific cancers[16,21,22]. As a miRNA target, full-length Dicer was also shown to be directly repressed by miR-103/107 and this repression enhanced breast cancer metastasis[23], whereas transcriptional induction of Dicer by Tap63 suppressed metastasis[24].

The nearly global decrease in miRNAs observed across a range of human tumors suggests that Dicer loss could be necessary for tumor progression. To better understand how cancer cells respond to loss of miRNA expression, Philip Sharp and collaborators[25] have characterized the effects of homozygous deletions of *Dicer1*-conditional alleles on the tumorigenicity of murine sarcoma cells and on the cellular phenotype of immortalized murine mesenchymal stem cells (MSCs). *Dicer1*-/- cells survived and proliferated without recovery of miRNA processing. Interestingly, their two models are mesenchymal, corroborating our results that show a repression of Dicer during the EMT process. Inactivation of p53,

a common feature in both the sarcoma and MSC models, may facilitate, or be indispensable for, viability in the absence of Dicer. p53 loss was shown to allow primary MEFs to bypass an immediate senescence phenotype induced by *Dicer1* loss[26].

miRNAS AND BREAST CANCER PLASTICITY

As a consequence of Dicer loss, tumors of epithelial origin should express more miRNAs than mesenchymal tumors. If we compare development and cancer progression, a parallel can be drawn between miRNA biogenesis during embryogenesis with repression of miRNAs synthesis in stem cells, and global miRNA loss during acquisition of cancer cell stemness. In embryonic zebrafish development, most miRNAs were expressed in a highly tissue-specific manner during segmentation and later stages, but not early in development. This suggests that they do not play a role in tissue fate establishment, but rather in differentiation or maintenance of tissue identity[27]. The link between deregulated miRNA expression and cancer has been well established, with miRNA profiling studies revealing distinct expression profiles in various cancers that could help in the diagnosis of these malignancies[28-30]. Only a small subset of specific oncogenic miRNAs has been found to be upregulated in cancer.

Different miRNA signatures have been identified in the different breast cancer subtypes. Do these signatures reflect cell lineage of origin? miRNAs have been implicated in the development of murine mammary gland, which showed seven distinct temporal clusters during mammopoiesis[10,31] Among them, miRNAs clusters overexpressed during puberty and gestation in normal tissue of mammary gland, for example the miR-17-92 cluster, have been shown to play a crucial role in breast cell proliferation and have also been found in aggressive cancers, such as the basal-like subtype[32]. As it has been found for other types of cancer, miR-21 appeared to predominantly act as an oncogene and its expression is inversely correlated with the tumor suppressor PTEN (Phosphatase and TENsin homolog) expression[33]. Another potent miRNA oncogene, miR-191 was positively regulated by estrogen and was shown to promote proliferation and invasion[34] miR-155 was also categorized as an oncomiR, as it was implicated in TGF-β-induced EMT, cell migration and invasion. Let-7 was the diametric opposite of miR-21, acting as a general tumor suppressor, and was found down-regulated in breast cancers. Let-7 has been described as a regulator of self-renewal and a pro-differentiation miRNA of breast cancer cells repressed by the Wnt-β-catenin pathway[35], targeting oncogenes including RAS, HMGA2 and MYC. miR-21, miR-155 and let-7 are involved early in tumorigenesis and were found deregulated in benign breast tumors[36]. In contrast to miR-191, miR-206 was negatively regulated by estrogens and decreased miR-206 levels are associated with breast cancer of advanced clinical stage and shorter overall survival. Different studies have profiled the expression of miRNAs as a function of intrinsic breast cancer subtype. A clear miRNAs signature was identified in luminal breast cancer, with overexpression of miR-191 and miR-26 and down-regulation of miR-206. Interestingly, based on miRNAs signatures, the tumors can be easily classified as luminal A, luminal B, normal-like, HER^{2+} and basal-like[32].

EMT-TFs signatures were more often found in Triple Negative Breast cancer (TNBC), a very aggressive cancer subtype representing, however, a very heterogeneous group of breast cancers with the only common phenotype being ER-, PR- and HER2-negative. Further transcriptomic studies allowed the sub-classification of TNBC by identifying additional entities such as the Claudin-low subtype, characterized by low expression of claudin proteins, proliferation genes and luminal markers, and high expression of EMT markers and CSC-like features[37]. Interestingly, as previously mentioned for epithelial-mesenchymal dichotomy of miRNA expression, the highly undifferentiated nature of TNBC is correlated with a global down-regulation of microRNAs[38]. However some miRNA are readily expressed in stem cells, such as the miR-302 cluster, "stemness miRNA cluster," in ES cells which decreases upon cell differentiation, and is undetectable in somatic cells. An Oct4/Sox2-miR-302-cyclin D1 regulatory network governing ES cell pluripotency and self-renewal properties has been proposed[39]. miR-302 over-expression converts cancer cells into ES-like pluripotent stem cells associated with high expression of Oct3/4, SSEA-3, SSEA-4, Sox2, and Nanog[40]. The group of Carlos Caldas also identified miR-301a as a hub of pluripotency in breast cancers, and demonstrated that mRNA relationships altered in miR-301a high/low tumors showed a link between immune and EMT pathways, illustrated by the immunoglobulin superfamily member ALCAM, the EMT-TF ZEB2 and Claudin-3. miR-301a directly targeted and suppressed the tumor suppressor PTEN, one negative regulator of the Wnt/β-catenin signaling cascade, which promotes breast cancer invasion and metastasis. In the Claudin-low subtype, while the global decreased miRNAs expression can be assigned to a repression of Dicer expression, the down-regulation of miRNAs targeting transcription factors implicated in EMT and cancer stem cells may result from a transcriptional repression of their promoters. The miRNAs targets can directly drive this repression (Figure 2).

FEEDBACK LOOPS INVOLVING miRNAS DURING EMT-MET

EMT is driven both by transcriptional and post-transcriptional changes. Because of the reversible nature of EMT, miRNAs functioning as co-repressors or co-activators are key players in this plasticity, specifically involved in regulation networks with EMT-TFs. miRNAs are categorized as either EMT-inducers or EMT-repressors, inversely involved in MET.

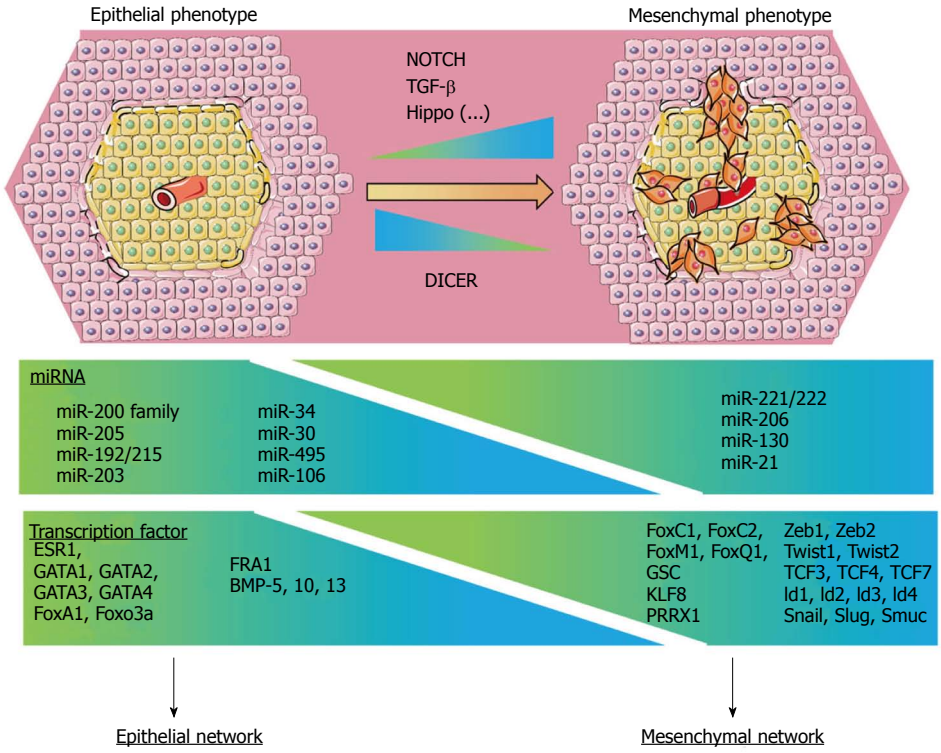

Figure 2 Transcription factors and miRNA epithelial and mesenchymal expression networks in breast cancer. Through activation of different signaling pathways such as TGF-β, Notch or Hyppo pathways and the down-regulation of Dicer, epithelial cells undergo the epithelial to mesenchymal transition. Transcription factors and miRNAs act together to be the "plastic surgeons" of the epithelial or mesenchymal phenotype. Regulation networks between these two main actors drive cells to plasticity.

miRNAs with EMT inducer activities

The well-known oncomiR miR-21 was identified as an EMT-inducer, similarly to miR-103/107 which represses Dicer and PTEN expression during breast tumor initiation. PTEN is a major miR-21 target that negatively regulates EMT and CSC phenotypes. miR-10b was also identified as a positive regulator of EMT as it was demonstrated to be a positive effector of TWIST. It was shown to induce migration and invasion capacities in breast cancer cells *via* the direct targeting of the HOXD10 transcript. HOXD10 is a known repressor of genes involved in cell migration and extracellular matrix remodeling, including RHOC, α3 integrin, matrix metalloproteinase-14 and urokinase-type plasminogen activator receptor[41].

The oncomiR miR-206 expressed in aggressive breast tumors is involved in a double-negative feedback loop with ERα and participates in EGFR-mediated abrogation of estrogenic responses in MCF-7 cells, thus contributing to a Luminal-A- to Basal-like phenotypic switch[42]. ERα is also involved in a simple negative feedback with the miR-18a (17-92 cluster), where ERα induced the expression of the mir-17-92 which in turn targets ERα with miR-18a. mir-17-92, an miRNA polycistron also known as oncomir-1, is among the most potent oncogenic miRNAs. Genomic amplification and elevated expression of mir-17-92 was found in several types of tumor, including mammary. mir-17-92 carries out pleiotropic functions during both normal development and malignant transformation, as it acts to promote proliferation, inhibit differentiation, increase angiogenesis, and sustain cell survival[43]. ERα functions in a forward positive feedback loop with miR-375. Inhibiting miR-375 in ERα-positive MCF-7 cells resulted in reduced ERα activation and cell proliferation. Researchers have identified RASD1 (Dexamethasone-induced Ras-related protein 1), a small G protein of the Ras family, as a potential miR-375 target. Mechanistic investigations revealed that miR-375 regulates RASD1 by targeting the RASD1 3'UTR and RASD1 negatively regulates ERα expression[44]. miR-206, which contributes to a Luminal-A- to Basal-like switch, targets KLF4 (Kruppel-like factor 4) a pivotal transcription factor that is associated with both tumor suppression and oncogenesis. In untransformed cells, KLF4 likely acts as a potent inhibitor of proliferation. Conversely, in transformed cells, KLF4 suppresses the expression of p53 by directly acting on its promoter; consistently, KLF4 depletion from breast cancer cells restores p53 levels and causes p53-dependent apoptosis[45]. To further complicate the function of KLF4, it was shown that a co-operative binding of KLF4 and p53 to the DNA binding sites of some p53 targets, contributes to p53 target selectivity[46]. miR-206 levels were KLF4-dependent in breast cancer cells, and a KLF4-miR-206 feedback pathway was identified that negatively regulates protein translation in normal cells and cancer cells[47]. Very recently, KLF4 was evoked in a feedback loop involving p21. The tumor suppressor p21 has been shown to regulate gene expression by func-

tioning as a transcription co-repressor. Li and collaborators[48], have identified p21-regulated miRNAs, among them, the miR-200 family and the miR-183-96-182 cluster, that were down-regulated in p21-deficient cells.

miRNAs with EMT repressor activities

miR-200 family members were identified as the guardians of the epithelial phenotype in many types of cancers, including breast cancers[49-51]. The miR-200 family activates the Sec23a-mediated tumor cell secretome which leads to secretion of metastasis-suppressive proteins. Predictably, loss of miRNA-200a is frequently observed in breast cancers, especially tumors with high-grade histology, but this loss does not predict tumor recurrence or patient survival[52]. miR-200 family members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) encoded from two clusters, directly target the mRNAs of the E-cadherin transcriptional repressors ZEB1 and ZEB2. Interestingly, Thomas Brabletz' group and others[50,53] have shown that both promoter regions are repressed in mesenchymal cells by ZEB1 and ZEB2 through their binding to a conserved pair of ZEB-type E-box elements, located proximal to the transcription start site. These findings establish a double-negative feedback loop controlling ZEB1-ZEB2 and miR-200 family expression. During EMT induced by TGF-β, miR200s are inhibited mainly by ZEB1[50]. The induction and maintenance of a stable mesenchymal phenotype requires the establishment of autocrine transforming growth factor-β (TGF-β) signaling to drive sustained ZEB expression. Prolonged autocrine TGF-β signaling induced reversible DNA methylation of the miR-200 loci, demonstrating the existence of an autocrine TGF-β/ZEB/miR-200 signaling network that regulates cancer cell plasticity[54]. But the activity of this miRNA family is a doubled edged sword during cancer progression, as it has been shown to promote MET through E-cadherin up-regulation, allowing migrating cancer cells to colonize distant tissues. Intriguing, the role of the miR-200 family during metastatic colonization can be partly elucidated by the oncogene c-Myb, which was shown to activate the expression of all five members of the miR-200 family. The transcriptional activation of miR-200 by c-Myb occurs through binding to myb binding sites located in the promoter regions of miR-200 genes on human chromosomes 1 and 12. Furthermore, when c-Myb and the transcriptional repressor ZEB1 are co-expressed, such as at the onset of EMT, the repression by ZEB1 prevails over the activation by c-Myb, and miR-200s are repressed. Researchers have also shown a positive correlation between the expression of c-Myb and miR-200 members in a dataset of breast cancer patients[55].

Interestingly, another EMT-TF, Slug (SNAI2, Snail2) is transcriptionally regulated by c-Myb and induces vimentin, fibronectin, and N-cadherin expression and membrane ruffling *via* actin polymerization, consistent with the acquisition of partial but not complete mesenchymal-like phenotype[56]. Both expression of c-myb and miR-200 members lead to simultaneous expression of vimentin, N and E-cadherin. These data support the concept that, during distinct phases of tumor progression, the role of the genes involved in the EMT process may change in relation to the expression of other regulators and to epigenetic changes. Are both mesenchymal and epithelial traits required for metastatic progression at distant sites? The complex relationship between miR-200 and ZEB during tumor progression was also investigated in a xenograft orthotopic model of breast cancer metastasis, where ectopic expression of members of the miR-200b/200c/429, but not the miR-141/200a, limits tumor cell invasion and metastasis. Despite modulation of the ZEB1-E-cadherin axis, restoration of ZEB1 in miR-200b-expressing cells was not sufficient to alter metastatic potential, suggesting that other targets contribute to this process[48].

Other feedback loops between EMT-TFs and miRNAs were identified during breast tumor progression. miR-183 and miR-96 repressed common targets, including Slug, ZEB1, and KLF4. Re-introduction of miR-200, miR-183 or miR-96 into p21-/- cells inhibited EMT, cell migration and invasion. p21 forms a complex with ZEB1 at the miR-183-96-182 cluster promoter to inhibit transcriptional repression of this cluster by ZEB1, suggesting a reciprocal feedback loop. ZEB1 and ZEB2 are also involved in a negative feedback loop with miR-205 through the E-box motifs present in the miR-205 promoter sequences[57]. During Snail-induced EMT in MCF7 breast cancer cells, miR-203 and miR-200 family members were repressed in a correlated manner. Importantly, miR-203 repressed endogenous Snail, forming a double negative miR-203/Snail feedback loop[58]. miR-203 is also able to target Slug (SNAI2). In parallel with the TGF-β/ZEB/miR-200 negative loop, TGF-β induced Slug to promote EMT by repressing the miR-203 promoter to inhibit its transcription. SNAI2 and miR-203 thus form a double negative feedback loop. It was found that miR-203 was significantly down-regulated in highly metastatic breast cancer cells, and the restoration of miR-203 in these cells inhibited tumor cell invasion *in vitro* and lung metastatic colonization *in vivo* by repressing Slug[59].

The miR-34 family is one of the most studied tumor suppressor miRNAs and comprises miR-34a, miR-34b and miR-34c. miR-34 is implicated in the inhibition of EMT mediated by p53. It was reported that activation of p53 down-regulates the EMT-inducing transcription factor Snail *via* induction of the miR-34a/b/c genes. Suppression of miR-34a/b/c caused up-regulation of Snail and EMT markers, and enhanced migration and invasion. Ectopic miR-34a induced MET and down-regulation of Snail. miR-34a also down-regulated Slug and ZEB1, as well as the stemness factors BMI1, CD44, CD133, OLFM4 and c-MYC. Conversely, the transcription factors Snail and ZEB1 bind to E-boxes in the miR-34a/b/c promoters, thereby repressing miR-34a/b/c expression. miR-34a prevents TGF-β-induced EMT, and the repression of miR-34 genes by Snail and related factors is part

of the EMT program[60]. miR-34 and SNAIL represent a double-negative feedback loop controlling cellular plasticity, governed by p53.

Transcription factors/miRNAs regulating networks

What are the targets of miRNAs during EMT/MET and what is the mode of TF-miRNA co-operation during pluripotency reprogramming? Sass *et al*[61] demonstrated that miRNAs which target the same protein complexes are frequently co-expressed. They experimentally verified that the miR141-200c cluster simultaneously targets several protein components of the CtBP (C-Term binding proteins)/ZEB complex (CtBP are conserved transcriptional co-repressors), implying an efficient regulation of a protein complex by a cluster of miRNAs. There is also evidence of functional redundancy among miRNAs resulting, in part, from miRNAs existing in large families sharing common seed sequences that can be co-expressed in the same cell. Redundancy also occurs at the level of co-targeting, where multiple distinct miRNAs with different sequences commonly target a single transcript through non-overlapping sites[62]. The miRNA-regulated protein complexes are mainly involved in regulation of transcription and chromatin modification. Conversely, house-keeping functions, such as translational elongation, are under-represented, meaning that miRNAs are "regulators of regulators"[61].

An important goal is to elucidate how complex TFs/miRNAs networks evolve in cancer. TFs and miRNAs are the two largest families of trans-acting, gene regulatory molecules in multicellular organisms, and they share a common regulatory logic. TFs generally do not work in isolation, but instead, together with co-regulators, they form large networks of co-operating and interacting transcription factors. The term "motif" was used to describe a small group that illustrates the regulation patterns of an miRNA, a TF, and their target genes. Common motifs, such as feedforward loops (FFLs) and feedback loops (FBLs) have been found to play crucial roles in cancer, such as the miR-17 cluster, E2F1, and c-Myc that modulate cellular proliferation[63]. However, the miRNA-TF synergistic effect may not be limited only to the FFLs or FBLs. Non-loop forms, such as the cascaded form, which have helped in understanding the regulatory mechanism, are also candidates[48]. miRNAs can also antagonize the function of other miRNAs, for example, miR-22 can suppress the expression of miR-200 *via* direct targeting of chromatin remodeling enzymes such as TET family members, which leads to the hypermethylation of the mir-200 promoter[64].

How can we decipher miRNA-regulating networks composed of proteins with opposite functions? For example, let-7 acts as a protective miRNA that inhibits RAS and transcriptional factors thus leading to cell commitment during development, but paradoxically Dicer, the master regulator of miRNA maturation is a hub for let-7 targeting. A recent finding may help us to understand this paradox; *ZEB2* transcript was shown to function as a competitive endogeneous RNA (ceRNA) for PTEN miRNAs. ZEB2 loss during MET can lead to repression of PTEN[65] and this regulation, that may appear counter-intuitive at first glance, may explain how MET could be intricately linked to stemness acquisition (Figure 3).

EMT, miRNAS AND CHEMORESISTANCE

Chemotherapeutics and radiotherapy effectively reduce tumor bulk but have little effect on cancer stem cells (CSC) that stimulate tumor recurrence, emphasizing the importance of identifying CSC-specific pathways that may be exploited to selectively target these resistant cells. Induction of EMT can activate some CSC state-specific signaling transcriptomic networks and the therapeutic resistance associated with CSCs. It was shown that EMT could be induced by chemotherapeutic agents and patients receiving neo-adjuvant therapy were more likely to express EMT-TFs in their circulating tumor cells (CTCs)[66]. Adriamycin treatment has been seen to induce EMT in a Twist-dependent manner in breast cancer cells. Additionally, irradiation, a common treatment modality in breast cancer, can increase EMT and CSC characteristics[67]. Tam and colleagues[68] found that EMT stimulated a switch between two main kinase pathways, through the protein kinase C α (PKCα). PKCα was activated following EMT by a shift from EGF receptor (EGFR) signaling, which predominated in non-CSCs, to autocrine platelet-derived growth factor receptor (PDGFR) signaling in mesenchymal stem-like cells and basal breast cancer cell lines. Up-regulation of PKCα resulted in induction of the transcription factor FRA1 (FOS-like antigen 1), which was required for CSC viability and FRA1 expression was directly induced by the EMT transcription factors TWIST and Snail in triple-negative breast cancer (TNBC).

The mechanism of action of miRNAs in drug-induced EMT remains mainly unknown. miR-21 up-regulation has been associated with taxol resistance in breast cancer cells[69] and suppression of the oncogenic miR-21 sensitizes cancer cells to chemotherapy. Results by Li and collaborators[70] reported that miR-448 is the most strongly down-regulated miRNA following chemotherapy. Suppression of miR-448 correlated with EMT induction in breast cancer *in vitro* and *in vivo*. miR-448 suppression induces increasing epidermal growth factor receptor (EGFR)-mediated TWIST1 expression, as well as nuclear factor κB (NF-κB) activation. The authors have also demonstrated that the adriamycin-activated NF-κB directly binds the miR-448 promoter, suppressing its expression, suggesting a positive feedback loop between NF-κB and miR-448. It was shown that the loss of miRNA-200c correlated with the acquired resistance of breast cancer cells to adriamycin[71]. In breast CSC, the Wnt-β-catenin pathway suppresses mature let-7 miRNAs by up-regulating Lin28, a negative let-7 biogenesis regulator. Loss of function of Lin28 impairs Wnt-β-catenin-pathway-mediated let-7 inhibition and breast cancer stem

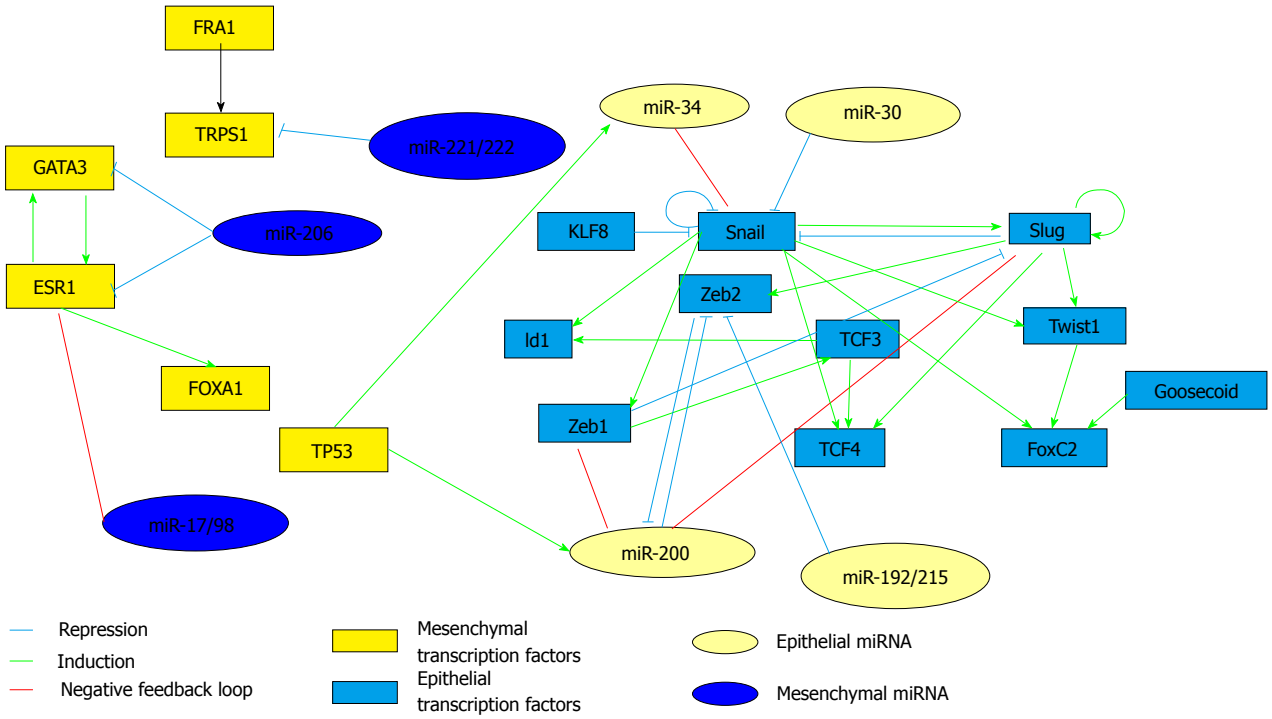

Figure 3 Feedback and feedforward loops existing between transcription factors and miRNA during breast cancer progression. Epithelial and mesenchymal regulators modulate their own expression creating regulatory feedback and feedforward loops.

cell expansion; enforced expression of let-7 blocks the Wnt-β-catenin pathway-stimulated breast CSC phenotype. Another study has shown that Lin28 expression was dramatically increased in tumor tissues after neoadjuvant chemotherapy, in local relapse and in metastatic breast cancer tissues[72].

CONCLUSION

Due to their implication in tumor development and metastasis, miRNAs represent potential therapeutics tools. Several studies either inhibiting or re-introducing miRNAs involved in EMT and CSCs regulation, are currently ongoing. Cai *et al*[35] have delivered a let-7a agomir into the pre-malignant mammary tissues of MMTV-wnt-1 mice and shown that it resulted in a complete rescue of the stem cell phenotype driven by the Wnt-β-catenin pathway. An interesting approach to neutralize miRNAs is to saturate them with target mRNAs. These artificial targets are called "miRNA sponges", expressing an mRNA containing multiple tandem binding sites for an endogenous miRNA and thus prevent the association of the miRNA with its endogenous targets[73]. We can hypothesize that introducing a ZEB2 miRNA sponge transcript in metastatic breast cancer cells may lead to a de-repression of PTEN transcripts.

In order to better evaluate the TFs--miRNAs regulatory relationships during mammary cancer progression, the next step is to identify specific combinations of epithelial and mesenchymal TFs-miRNAs networks coexisting in metastatic and stem-like cells in breast cancers. It is crucial to discriminate between the aberrant dynamics of epithelial-mesenchymal transitions during tumorigenesis and normal programs of embryonic development and wound healing. Interestingly, a synthetic analysis has shown that a core regulatory unit composed of two highly interconnected modules, the miR-34/SNAIL and the miR-200/ZEB double negative feedback loops, participate in the regulation of stemness, genome stability, cell-cell communication, and cellular motility. The authors have shown that the miR-200/ZEB loop exhibits tristability (the existence of three distinct stable states: epithelial, hybrid and mesenchymal) and that the miR-34/Snail circuit exhibits monostability (existence of a single stable state)[74]. Regarding breast tumorigenesis, the miR-200/ZEB circuit is likely involved in cell plasticity and the miR-34/Snail in the stabilization of metastatic phenotype.

Manipulation of the EMT-TF-miRNAs feedforward and/or feedback loops may provide new therapeutic targets for breast cancers.

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