

# The network of pluripotency, epithelial–mesenchymal transition, and prognosis of breast cancer

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**Abstract:** Breast cancer is the leading female cancer in terms of prevalence. Progress in molecular biology has brought forward a better understanding of its pathogenesis that has led to better prognostication and treatment. Subtypes of breast cancer have been identified at the genomic level and guide therapeutic decisions based on their biology and the expected benefit from various interventions. Despite this progress, a significant percentage of patients die from their disease and further improvements are needed. The cancer stem cell theory and the epithelial–mesenchymal transition are two comparatively novel concepts that have been introduced in the area of cancer research and are actively investigated. Both processes have their physiologic roots in normal development and common mediators have begun to surface. This review discusses the associations of these networks as a prognostic framework in breast cancer.

**Keywords:** stem cells, epithelial-to-mesenchymal transition, mesenchymal-to-epithelial transition

## Introduction

Breast cancer is the most common malignancy of women and affects approximately one in eight women during their lifetime. Breast cancer is a heterogeneous disease, and prognosis may vary significantly in individual patients. Based on the expression of a few proteins by the cancer cells, estrogen receptor (ER), progesterone receptor (PR), Her2/Neu, and Ki67 antigen, breast cancers are divided into subtypes with clinical prognostic and therapeutic implications. These subtypes correlate well with genomic profiles that have been determined by whole-genome techniques.<sup>1</sup> ER-positive breast cancers are the most common, representing approximately three-quarters of the total number of cases, and may also express PR to various degrees. They are further divided into two subtypes based on low or high expression of Ki67 antigen. These subtypes correspond to the genomically defined subtypes luminal A and luminal B, respectively. The remaining one-quarter of breast cancers is shared by two other subtypes, the Her2/Neu-overexpressing and the so-called triple-negative (not expressing ER, PR, or Her2/Neu). The clinical, prognostic, and therapeutic implications of breast cancer subtypes are well known. Hormone receptor-positive luminal cancers metastasize more often to bones, while Her2/Neu-overexpressing cancers tend to favor the liver, brain, and lungs, and triple-negative cancers metastasize more often to lungs and brain.<sup>2</sup> Her2/Neu-overexpressing and triple-negative cancers tend to have a worse prognosis but respond better to chemotherapy than the luminal types.<sup>3</sup> In contrast, luminal cancers are the only ones that respond to hormonal treatments, albeit to various degrees, with luminal B subtype being less hormone-sensitive, at least in the long run.<sup>4</sup> Despite the

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ability to prognosticate along the above broad lines in clinical practice, a significant amount of uncertainty remains in determining prognosis of the individual cancer patient, a fact that impedes individualized therapeutic decisions. Thus, further efforts to improve prognosis determination in breast cancer would be helpful in the clinic. These would further refine the current prognostic classification and would be particularly useful for luminal and triple-negative cancers where it is evident that there exists significant intra-category variation. This paper will discuss two important cancer cell processes, pluripotency and epithelial–mesenchymal transition (EMT); their relationship; and the ability of proteins involved in them to predict prognosis in breast cancer patients.

## Cancer stem cells

Normal adult tissues possess a small percentage of cells that retain the potential to self-renew and differentiate to cell types of their resident organ. These cells are called stem cells and serve in cell replacement and organ regeneration during the adult organism lifespan.<sup>5</sup> Stem cells expressing pluripotency proteins have been identified in the normal human breast.<sup>6</sup>

According to the cancer stem cell hypothesis, cancer stem cells (or tumor-initiating cells), representing usually a small percentage of the total cancer cell population, are at the basis of tumor development and propagation. Cancer stem cells have properties of normal tissue stem cells and may derive from malignant transformation of these cells or from transformation of differentiated cells that acquire mutations allowing dedifferentiation and acquisition of tumor-initiating properties. Tumor-initiating properties are defined experimentally by the ability of the cells in question to be serially passaged in immunosuppressed mice and produce tumors, with heterogeneity of cells observed in the initial tumor. They are able to do so even when transplanted in mice at much lower numbers than the bulk of the tumor cells.<sup>7,8</sup> The derivation of tumor stem cells from normal stem cells or differentiated cells is not mutually exclusive, and either may be true in different patients. In addition, it may explain the different subtypes, as neoplastic stem cells may retain epigenetic memory of their initial cell of provenance and differentiate back to it when producing the bulk cell population. For example, in breast cancer, such considerations would explain how transformation taking place in diverse normal cells would give rise to the different cancer phenotypes. Derivation from a normal stem cell may produce triple-negative cancers, while derivation from dedifferentiation of ductal cells may give rise to luminal subtypes. Stem cells have the phenotype CD44<sup>high</sup>/CD24<sup>low</sup>

in human breast cancers.<sup>7</sup> As few as 200 cells with this phenotype reconstitute tumors when implanted in mice, while more than  $50 \times 10^3$  unsorted cells are needed.<sup>7</sup> Experimental work has attempted to capture the provenance of different subtypes of breast cancer from different normal breast epithelial cells.<sup>9</sup> Although more prevalent and easily identifiable by immunohistochemistry in triple-negative tumors, CD44<sup>+</sup>/CD24<sup>–</sup> cancer stem cells are common for all breast cancer subtypes.<sup>10</sup>

Transformed (cancer) stem cells contain mutations that lead to the acquisition of additional properties on top of the tumor-initiating cells' properties described above which are essentially properties of normal tissue stem cells. These mutations endow them with the required abilities for cancer development and maintenance, including sustained proliferation and evasion from growth suppressors, resistance to apoptosis, induction of angiogenesis, invasion and metastasis potential, genome instability, and avoidance of immune recognition and destruction.<sup>11</sup> Activating mutations in oncogenes or debilitating mutations in tumor suppressor genes are predicted to be stronger cancer promoters if they can establish the stem cell network and concomitantly favor the acquisition of cancer capabilities. Cancer capabilities overlap partially with stem cell properties but are no longer regulated by extrinsic signals. The presence of the survival-promoting pluripotency network in stem (or stem cell-like) cells would allow for a requirement of less additional genetic lesions in order for a cancer to be established.

Given that normal tissue and cancer stem cells have the potential to reconstitute the whole variety of the cells in their organ or of the bulk tumor cell population, they are characterized by plasticity reminiscent of pluripotent embryonic cells that can produce diverse tissues of the three embryonic layers (endoderm, mesoderm, and ectoderm) in development. Pluripotency is conferred by a network of pluripotency transcription factors and microRNAs (miRs), which have also been found to induce a state of pluripotency when exogenously expressed in differentiated cells (so-called induced pluripotent stem cells).<sup>12–14</sup> Thus, pluripotency networks may be expected to be in place in cancer stem cells in order to endow them with the ability to self-renew indefinitely and to differentiate toward phenotypically distinct cells of the tumor tissue.

One of the programs present in stem cells of tissues and their embryonic and cancerous counterparts is EMT. EMT and the reverse process, mesenchymal–epithelial transition (MET), are developmentally derived programs that are reactivated in cancers and facilitate the acquisition of cancer properties such as invasiveness, motility, and metastasis.<sup>15</sup>

The embryonal pluripotency network and EMT are intertwined in normal development and in breast cancer cells.<sup>16,17</sup> Given the importance of both processes in cancer and their relationship with each other and with stem cell properties, they deserve to be investigated as markers in breast cancer prognosis.

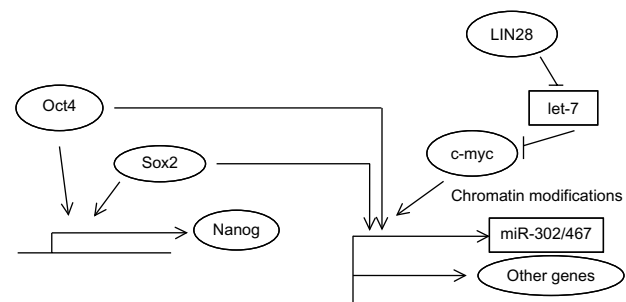
## The network of pluripotency transcription factors and miRs and their expression in breast cancer

The pluripotency state, the ability of cells to remain undifferentiated but at the same time to retain the potential of differentiation to multiple tissues is obtained through the function of defined combinations of a few transcription factors and miRs that work together, establishing a network. These transcription factors include Oct4, Nanog, Sry containing box 2 (Sox2), Krüppel-like factor 4 (KLF4), LIN28, and c-myc. The miR-372/373 cluster of miRs also participates in pluripotency networks and has been reported to enable the induction of pluripotency without the need for additional transcription factors.<sup>18</sup> Transcription factors of pluripotency and miRs are involved in establishing the pluripotency state during development in embryonic cells and during the backward reprogramming of adult differentiated cells, termed induced pluripotent stem cells, that have recently been obtained experimentally.<sup>12,13</sup> Transcription factors of the network often work in concert on promoters of target genes to establish and maintain pluripotency, a characteristic of embryonic and immature cells that may differentiate toward any cell of the three embryonic layers. Besides inhibiting differentiation, the network of pluripotency promotes cell survival to ensure embryonic development.<sup>19</sup> In addition, this program could be usurped by cancer stem cells which display a similarity to normal embryonal pluripotent cells and induced pluripotent stem cells in that they can reconstitute the tumor tissue. A discussion of transcription factors and miRs involved in pluripotency establishment and their expression in breast cancer follows in this section.

Oct4 (alternatively named Oct3/4, OTF3, or POU5F1) is a homeodomain transcription factor of the POU family. It binds to DNA sequences through its POU domain.<sup>20</sup> Its action in embryonic stem cells (ESCs) ensures their survival and maintenance of their pluripotentiality.<sup>21</sup> These effects are accomplished in cooperation with other pluripotency transcription factors such as, notably, Sox2.<sup>22</sup> Oct4 is downregulated by promoter methylation and its expression is suppressed in differentiated adult tissues.<sup>23</sup> Various posttranslational modifications are important in controlling

Oct4 activity. Phosphorylation of Oct4 by kinase Akt facilitates its interaction with Sox2 in promoters of target genes such as Oct4 itself and Nanog (Figure 1).<sup>24</sup> In addition, this phosphorylation inhibits ubiquitination of Oct4 that leads to degradation by the proteasome and promotes dissociation of the unphosphorylated Oct4 from the Akt promoter where it acts as a transcription suppressor.<sup>24</sup> Akt has a key position in transduction cascades downstream of several cell surface growth factors and, importantly for breast cancer, downstream of Her2/Neu. Regulation of Oct4 by Akt is only one example of how signaling cascades emanating from the cell surface affect pluripotency. Oct4 is expressed in less than 1% of cells in normal breast epithelium, but its expression is detected in 11.4% of cells in the lactating breast and further increases to more than 30% of cells in breast cancers diagnosed during lactation.<sup>25</sup> These data support the role of Oct4 in breast physiology during a phase of generation of functional lactation units that is usurped in breast cancer.

Sox2 is a transcription factor encoded by an intronless gene at human chromosome 3q26. It belongs to the Sox family, group SoxB1 (together with Sox1 and Sox3), and has a length of 317 amino acids.<sup>26,27</sup> Sox factors display a high conservation along species.<sup>28</sup> Sox2 binds to DNA through its HMG domain, and this binding leads to stereotactic repositioning of the minor groove of the DNA helix to allow accommodation of the transcription machinery.<sup>26</sup> Besides being essential for epiblast development in early mammalian embryo, Sox2 plays a part in the maintenance of ESCs and progenitor cells in many tissues. Another common characteristic of Sox factors is that in general they do not elicit a transcription response alone, but depend on binding of partner transcription factors in adjacent DNA sites.<sup>29</sup> Notable partners are Oct4 and Nanog, with several



**Figure 1** Schematic representation of the cooperative action of pluripotency transcription factors on promoters of target genes.

**Notes:** Oct4 and Sox2 often work in synergy to transcribe genes such as Nanog which then cooperate with them on additional targets. c-myc facilitates the process by helping create permissive chromatin modifications. LIN28 neutralizes the inhibitory effect of mRNA let-7 on c-myc. Arrows denote activation and — denotes inhibition.

hundred promoters bound together by these factors in ESCs when studied by ChIP-on-chip or ChIP-seq.<sup>30,31</sup> MCF-7 breast cancer cell line fraction with high Sox2 promoter activity (denoting high Sox2 expression) displays a high sphere-forming capacity and high expressions of CD44 and Nanog compared to the fraction with low Sox2 promoter activity.<sup>32</sup> Sox2 is expressed in early breast cancer samples studied by immunohistochemistry.<sup>33</sup> Its increased expression confers enhanced mammosphere formation in breast cancer cells in vitro, while knockdown of Sox2 prevented mammosphere formation and delayed xenograft tumor formation in mice.<sup>33</sup> Another study confirmed Sox2 expression in a significant proportion of breast cancer pathology samples across tumor subtypes.<sup>34</sup>

Nanog is another core pluripotency network member. Similarly to Sox2, it is conserved across species.<sup>35</sup> Nanog is a 305 amino acid transcription factor possessing a homeobox domain located centrally in the protein through which it binds DNA.<sup>36</sup> It activates transcription through two transactivation domains located in the carboxy-terminus of the protein.<sup>36</sup> Nanog has important effects in modulating the Oct4 transcriptome, and this translates to the transition phase of reprogramming leading from dedifferentiation to pluripotency.<sup>37</sup> This is also due in part to its acting as a pioneer factor for Oct4.<sup>38</sup> Pioneer factors are proteins bound to DNA that promote the interaction of partner transcription factors with subsets of their target promoters in a particular cell context where they are expressed. Nanog is also a target of the Oct4/Sox2 module of transcription and then participates with these two transcription factors in the activation of several target gene promoters (Figure 1).<sup>39</sup> In breast cancer cells, although Nanog transfection by itself is not sufficient to transform cells, it can cooperate with other pathways to this effect.<sup>40</sup> Forced expression of Nanog enhances proliferation of breast cancer cells while knockdown of Nanog inhibits them.<sup>41</sup>

All three transcription factors, Oct4, Sox2, and Nanog, are expressed in breast cancer cell lines, albeit to differing degrees.<sup>42</sup> Nanog protein expression has been confirmed in the side population of the MCF-7 cell line,<sup>43</sup> while another study showed that both Oct4 and Nanog were expressed in human breast carcinomas, in contrast to normal breast epithelial cells.<sup>44</sup>

c-myc is a basic helix-loop-helix (bHLH) transcription factor with a role in neoplastic transformation. The protein binds to DNA through its carboxyterminal DNA binding domain. The target DNA sequence is known as E-box and has the consensus CANNTG (where N is any nucleotide).<sup>45</sup> c-myc was included in one of the gene factor “cocktails” that may

reprogram adult cells into pluripotent stem cells.<sup>46</sup> Mechanistically, c-myc contributes to pluripotency induction by promoting chromatin modifications that subsequently permit binding of additional factors (Figure 1). Moreover, its well-known effect in cell cycle promotion may favor the selection of rare cells in a population primed to become pluripotent.<sup>47</sup> These mechanistic insights corroborate with the fact that there are actually no known genes that are regulated solely by c-myc and, on the other hand, approximately 15%–20% of vertebrate genes are regulated by c-myc.<sup>48</sup> c-myc is involved in normal breast physiology, where it contributes to lobular proliferation and milk production during lactation.<sup>49</sup> Its role in normal mammary progenitor cell maintenance is highlighted by the decreased ability of mutant glands (with a disabling mutation) to reconstitute mammary glands in experiments with cleared mammary fat pads in mice.<sup>49</sup> The role of c-myc was extensively studied in breast cancer before its role in stem cell networks became known.<sup>50</sup> c-myc is activated by ER $\alpha$ , Her2/Neu, and oncogene K-Ras downstream of it, as well as by the Wnt and Notch pathways. ER $\alpha$  and Her2/Neu are breast cancer-specific oncogenes activated in defined subsets, while the Wnt and Notch pathways are involved in development but also in carcinogenesis. In addition to being a target of  $\beta$ -catenin transcription in the Wnt pathway, c-myc provides a feedforward activation of the pathway by inhibiting the transcription of two inhibitors of Wnt signaling, DKK1 and SFRP1.<sup>51</sup> C-myc protein is expressed in most clinical breast cancer specimens, as shown in a breast cancer patient series.<sup>52</sup> Another evaluation using tumor microarrays from Her2/Neu-positive patients that had participated in a randomized trial showed high (more than 30% of cells) nuclear c-myc staining in 33% of patients and intermediate staining (10%–30% of cells) in 28% of patients, while 38% of patients had low (less than 10% of cells) nuclear c-myc staining.<sup>53</sup> Nuclear staining correlated with cytoplasmic staining of c-myc.

KLF4 is a zinc finger transcription factor included in combinations of transcription factors able to induce pluripotency.<sup>46</sup> The 470 amino acid KLF4 protein recognizes a GC-rich sequence that contains a CACCC element and an element called BTE (basic transcription element).<sup>54</sup> In the fetus, KLF4 expression peaks in later parts of development, while in the adult organism it is expressed in skin, lung, and intestinal tissues. In breast cancer cells from tumors in transgenic mice and in human breast cancer cell lines, KLF4 is expressed in higher levels in the stem cell subset compared with the bulk cell population.<sup>55</sup> Knockdown of KLF4 by shRNA decreased the cancer stem cell population percentage while, in contrast, forced expression of the



protein increased the stem cell population.<sup>55</sup> In addition, KLF4 promotes cytokine production in breast tumor cells, resulting in tumor progression through modifications of the tumor immune microenvironment.<sup>56</sup>

LIN28A and LIN28B are two paralogous RNA-binding proteins that are important regulators of development and pluripotency promotion,<sup>57</sup> mainly by blocking the biogenesis of the differentiation-inducing let-7 family of miRs (Figure 1). let-7 family members target mRNAs such as LIN28 itself (establishing a double-negative feedback loop), c-myc, Sall4, interleukin 6, and K-Ras mRNAs.<sup>58</sup> In breast cancer cells, LIN28 is upregulated by the Wnt/ $\beta$ -catenin/LEF1 pathway and suppresses let-7 to expand stem cells (Figure 1).<sup>59</sup> The two LIN28 paralogs characterize different subsets of breast cancers, with LIN28A mostly expressed in Her2-positive cancers and LIN28B expressed predominantly in triple-negative cancers.<sup>60</sup>

In addition to the let-7 family, three other human miR families, the miR-372/373, the miR-302/467, and the miR-17-92 clusters, participate in the establishment of pluripotency. These clusters cooperate in very early embryonic development when pluripotency is established in the zygote after fertilization.<sup>61</sup> The miR-302/467 cluster is a target of Oct4 and of c-myc transcription (Figure 1)<sup>62,63</sup> and is able to reprogram human and mouse fibroblasts to pluripotency without transcription factors.<sup>18</sup> Reprogramming using miR transfection arises as a more efficient method than using transfection with the Oct4/Sox2/KLF4/c-myc combination and does not require any additional factors, at least in human fibroblasts. Oct4 is upregulated by miR-302/467 cluster transfection, establishing a feedforward loop of pluripotency. In breast cancer, miR-302a downregulation has been associated with radio-resistance of unfractionated cells, and re-expression of the cluster was found to increase cell radio-sensitivity in vitro and in vivo in mice.<sup>64</sup> miR-302a downregulation allowed higher expression of AKT1 and RAD52 proteins. Other members of the cluster were not changed in radiosensitive and resistant cells.<sup>64</sup> This is in direct contrast to human head and neck cancer stem cells, where miR-302 induced by Oct4/Sox2/Nanog, following interaction with the ligated hyaluronan receptor variant CD44v3, induced increased clonogenicity and cisplatin resistance.<sup>65</sup> Although these results are difficult to reconcile, it is interesting to note that the breast cancer cell experiments were carried out with phenotypically radio-resistant bulk cell lines without sorting. In contrast, the head and neck experiments, as mentioned, referred specifically to cells expressing the stem cell marker CD44v3.

The pluripotency-inducing miR-17-92 cluster is a target of induction by c-myc.<sup>66</sup> Members of the cluster and of the paralogous cluster miR-106b-25 target PI3K pathway inhibitor PTEN, factors of the E2F transcription factor family, proteins of the TGF $\beta$  pathway, and the apoptotic bcl-2 family member BIM.<sup>67</sup> These downregulations would lead to cell cycle and apoptosis inhibition, PI3K pathway activation, and inhibition of EMT. The effect on TGF $\beta$  pathway signaling and EMT is more complex, because the miR-106b-25 cluster has been reported to activate this signaling by targeting the inhibitor of the pathway Smad7.<sup>68</sup> Members of the cluster have been reported to be upregulated in triple-negative breast cancers but also in ER-positive cancers where they target ER $\alpha$  mRNA.<sup>69,70</sup>

The above data paint a picture of the complexity of the network of pluripotency in general and in breast cancer in particular. Several transcription factors cooperate to establish this network, although, certainly, not all of them may be necessary at one time or in a particular cancer case. In addition, an optimal range in the level of expression of several factors is necessary for the operative establishment of the network and higher levels may paradoxically promote differentiation as is seen with Oct4 in ESCs.<sup>71</sup>

## EMT factors and expression in breast cancer

EMT is a process during which an epithelial cell ceases to be part of an epithelial membrane, invades through the dissolving basement membrane, and moves on to the sub-epithelium of the tissue or further to distant organs. Several events take place in a cell undergoing EMT, including junction dissolution, loss of epithelial cell polarity, acquisition of a fibroblast-like shape, downregulation of epithelial markers, and upregulation of mesenchymal markers.<sup>72</sup> EMT is a process that physiologically takes place during normal embryonic development and in adult tissue injury repair. In contrast to these two physiologic conditions where EMT serves normal functions, cancer is a pathologic condition where EMT takes place. Cancer cells undergoing EMT upregulate specific mesenchymal markers such as S100A4 (also called FSP1 [fibroblast-specific protein 1]), vimentin, type I collagen and its receptor kinase DDR2 (discoidin domain receptor tyrosine kinase 2), and cadherin N.  $\beta$ -catenin moves to the nucleus during EMT, and downregulation of epithelial adhesion proteins such as E-cadherin, zonula occludens 1 (ZO-1), cytokeratins, claudins, occludins, and basement membrane components collagen IV and laminin 1 ensues.<sup>73</sup>

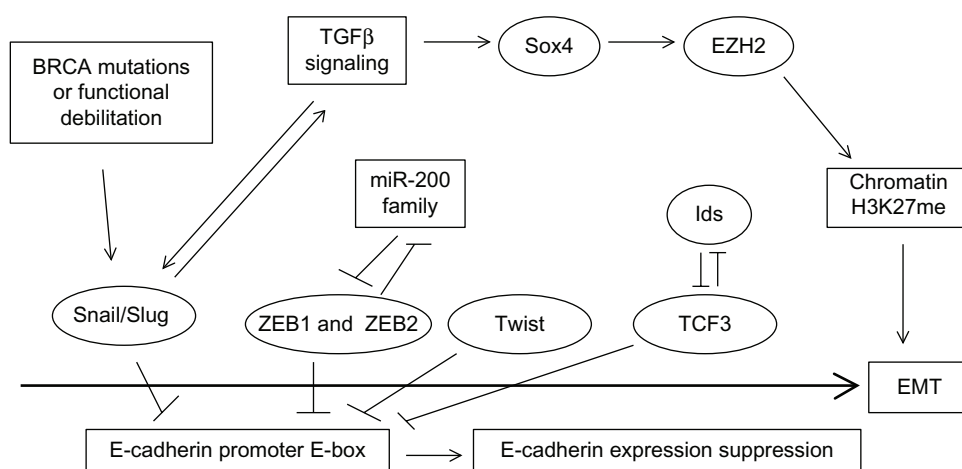
EMT occurring in cancer may be incomplete both at the individual-cell and cell-population level, and only some of the EMT markers may be expressed in small subsets of cancer cells.<sup>74</sup> Moreover, incomplete EMT, as, for example, seen during the process of collective migration, allows cells to detach from the epithelial site and acquire some mesenchymal features but still move as small groups of few cells without losing adhesions between the members of the group.<sup>15</sup> Further evidence for the role of EMT as intrinsic to the malignant process is provided by the discovery that beyond specific EMT-inducing factors, such as the core EMT transcription factors Snail and Slug (also called Snail2), a multitude of general cancer regulating pathways are important EMT regulators. Examples specifically pertinent to breast cancer include ER $\alpha$ , BRCA1, and p53 proteins, which will be discussed in later sections. Furthermore, confirmation of EMT interconnection with pluripotency networks (which will also be further discussed in a later section) highlights the role of both as cancer-intrinsic processes. In parallel with the above discoveries and related to the importance of the incompleteness of the EMT process in cancer and the associated presence of pluripotency features, it has now become clear that the reverse process of MET is of equal importance for metastasis establishment in remote organs.<sup>75</sup>

Several pathways activated in cancer have the ability to activate a set of core EMT transcription regulators which eventually lead to E-cadherin downregulation and cell–cell adhesion dissolution. EMT core factors include Snail1 and Slug, ZEB1 (also known as TCF8 or  $\delta$ EF1) and ZEB2 (also known as SIP1 or Zfhx16), Twist, and TCF3

(also known as E47). TGF $\beta$  signaling is prominent in inducing EMT core transcription factors and EMT in breast cancer<sup>76</sup> and, reciprocally, Snail and Slug induce components of the TGF $\beta$  pathway that contribute to the invasion component of EMT and establish a feedforward loop.<sup>77</sup> TGF $\beta$  signaling activates an additional EMT promoter in breast cancer cells, Sox4 (member of the Sox C Sox factors group), which works through regulation of the Polycomb repressive complex 2 (PRC2) histone methyltransferase EZH2 (enhancer of zeste homologue 2).<sup>78</sup> EZH2 executes the methylation of lysine 27 in histone 3, a marker of chromatin primed for transcription, involved in both EMT and pluripotency (Figure 2).

In breast cancer, the expression of EMT core factors supports various phases of EMT. For example, Snail1 is more important in the initiation phase, while Twist1 becomes essential later in the process.<sup>79</sup> ZEB transcription factors are also later players required for EMT maintenance.<sup>80</sup> EMT transcription factors have been most extensively studied in the basal-like subtype associated with BRCA1 mutations and, importantly, these mutations have been found to increase Slug stability (Figure 2).<sup>81</sup> A brief discussion of EMT core transcription factors and their expression in breast cancer follows.

Snail1 and Slug are zinc finger-containing transcription factors of the C2H2 type (similarly to the previously discussed KLF4 pluripotency factor) that promote EMT by suppressing E-cadherin and other adhesion molecules. Snail1 is a more potent E-cadherin suppressor. Both proteins bind E-box sequences with the consensus 5'-CANNTG-3' in the promoter of E-cadherin gene with their zinc fingers.<sup>82</sup>



**Figure 2** The road to EMT.

**Notes:** EMT core transcription factors such as Snail and Slug, ZEB1 and ZEB2, Twist, and TCF3 cooperate to establish EMT by suppressing E-cadherin and other actions. They are under control of multiple regulators that activate or inhibit them. Examples of these regulatory factors are depicted. Arrows denote activation and  $\vdash$  denotes inhibition. The thick arrow pointing to EMT illustrates that the process is progressive, with several factors acting along the way, as discussed in the text.

**Abbreviation:** EMT, epithelial–mesenchymal transition.

Both Snail proteins as well as TCF3 are expressed in the branching sites during normal mammary morphogenesis and are necessary and sufficient for induction of the process.<sup>83</sup> Snail1 and Slug protect cells in mammary tubules undergoing branching from apoptosis induced by p53 and BID.<sup>83</sup> In addition, they promote estrogen production that supports ductal outgrowth by inducing expression of the aromatase enzyme.<sup>82</sup> Snail1 also protects non-transformed human mammary epithelial cells from anoikis.<sup>84</sup> Snail1 inhibition, on the other hand, correlates with downregulation of RhoA, a GTPase which promotes motility.<sup>85</sup> Snail1 mRNA is expressed in a significant percentage (30%) of breast cancer specimens.<sup>86</sup> Another study showed that both Snail1 and Slug proteins are expressed in higher levels in breast cancer tissues than in surrounding normal breast tissues.<sup>87</sup> A report examining Snail expression across breast cancer subtypes found this transcription regulator to be overexpressed (defined as expression in more than 5% of cells) in 13% of ER-positive cancers and in 64% of ER-negative cancers.<sup>88</sup>

ZEB1 and ZEB2 are zinc finger transcription regulators that contain a homeodomain flanked by two zinc finger domains. Similarly to the two Snail proteins, they bind to DNA through E-boxes of target gene promoters such as E-cadherin, promoting EMT.<sup>89</sup> Their function is inhibited by the miR-200 family of miRs, which bind multiple sites on the 3'-UTRs of their mRNAs and promote the inverse process, MET. There exists a double-negative feedback loop, as ZEB1 binds E-boxes in miR-200 promoters and suppresses their transcription (Figure 2). In addition, miR-200 family miRNAs are critical mediators of MET required during reprogramming to pluripotency and are targets of the Oct4/Sox2 couple of factors.<sup>90</sup> In the breast cancer metastatic process, expression of ZEB transcription regulators has broader results than those conferred solely by suppression of E-cadherin, through suppression of the miR-200 family of miRs. When released from ZEB suppression, these miRs actively promote the reverse process, MET, in the metastatic site, at least partly, through suppression of transcription of secretome factor Sec23a.<sup>91</sup> As a result of this suppression, secretion of proteins Igfbp4 and Tinagl1 is suppressed from tumor cells arriving in a metastatic site. These proteins contribute antimetastatic colonization signals to the stroma cells of the metastasis target organ and their suppression has the end result that the host organ of the metastasis may not mount a metastasis-inhibiting signal.<sup>91</sup>

Twist is a bHLH transcription factor with a role in mammary development.<sup>82</sup> Twist molecules bind E-boxes in target gene promoters as dimmers. Twist1 was detected in a small

subset (1%) of patients with breast cancer in circulating tumor cells.<sup>92</sup> A study of the expression of Twist in breast cancer patients showed overexpression in approximately half of the patients.<sup>93</sup> A similar percentage of the patients in this study showed high expressions of Snail and Slug.

TCF3, another bHLH transcription factor, is able to induce EMT in breast cancer cells by directly suppressing E-cadherin through binding to E-boxes of the promoter called E-pal and E3.<sup>94</sup> The HLH factors of the Id family lacking a basic domain are inhibitors of TCF3 and modulate its effects on EMT creation (Figure 2). Despite this inhibition and the fact that TCF3 has been found to bind the E-cadherin promoter alone, Id proteins contribute to EMT maintenance in breast cancer and are expressed, together with TCF3, in human breast cancer samples, with a higher expression in the basal subtype compared to the luminal subtype.<sup>94</sup> Similarly, in another study using a different antibody, Id expression was noted in several metaplastic breast carcinomas but not in carcinomas with the “usual” morphology.<sup>95</sup> These data imply that an optimal level of TCF3 activity is required for maintenance of EMT, and Id proteins participate in the regulation of this activity.

The above data as a whole confirm that EMT core factors are expressed in subsets of breast carcinoma cells in human tumors and support the presence and importance of the EMT process in mammary carcinogenesis.

## The pluripotency–EMT/MET connection in breast cancer

Stem cells are innately endowed with a transcriptional program that promotes their plasticity. As a result, they can undergo EMT and the reverse MET process. The two programs, pluripotency and EMT, are intertwined in normal mammary epithelium. Slug, for example, expressed in a subpopulation of basal cells of the duct, besides regulating EMT, is involved in enhanced mammosphere growth and suppression of apoptosis.<sup>96</sup> After neoplastic transformation, EMT endows cancer stem cells with invasion and metastasis potential and MET endows them with the ability to establish metastases in remote organs.

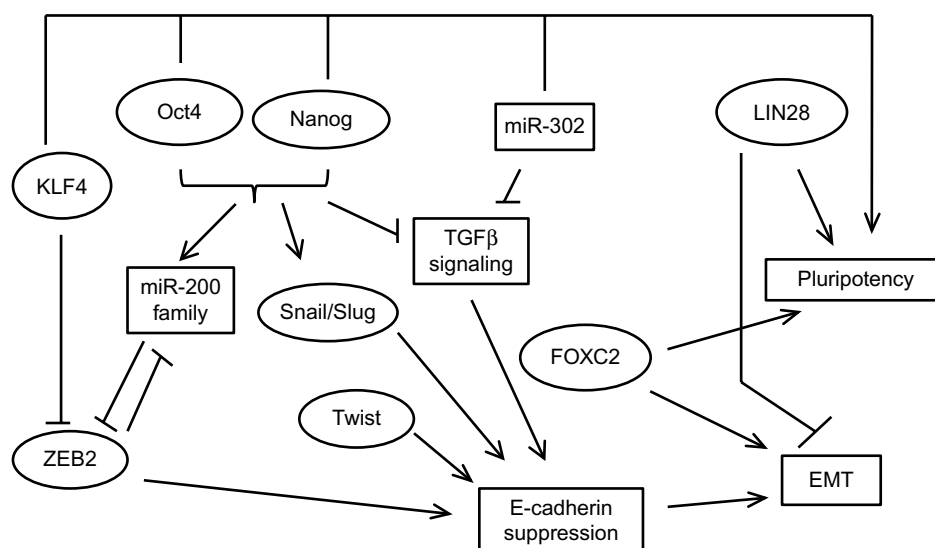
A direct link between EMT and gain of stem cell markers and properties has been experimentally shown in breast cancer.<sup>16,17</sup> When Snail or Twist were ectopically expressed in immortalized human mammary epithelial cells, these cells acquired a fibroblast-like, mesenchymal appearance; downregulated E-cadherin; and upregulated vimentin, N-cadherin, and fibronectin. In addition, they acquired a CD44<sup>high</sup>/CD24<sup>low</sup> phenotype consistent with the phenotype

of normal and cancer mammary stem cells and displayed an increased ability to form mammospheres, a characteristic of stem cells.<sup>16</sup> Reciprocally, endogenous cells with the stem cell phenotype  $CD44^{\text{high}}/CD24^{\text{low}}$  display a fibroblast-like morphology in culture and expression of mesenchymal markers. These results have been confirmed by using overexpression of Twist2 in non-transformed mammary cells and breast cancer cells.<sup>97</sup> In addition, as few as 20  $CD44^{\text{high}}/CD24^{\text{low}}$  breast cancer stem cells produced by overexpressing Twist are able to form tumors in immunocompromised mice.<sup>98</sup> A mechanism of pluripotency promotion by Twist has been proposed in head and neck squamous cell carcinoma cells and involves upregulation of the RING finger protein BMI1 by Twist.<sup>99</sup> Resistance to apoptosis in neoplastic cells undergoing EMT is accompanied by the acquisition of a stem cell phenotype also associated with drug resistance.<sup>16</sup> Another group reported that transfection of the human mammary cell line MCF-10A with an oncogenic mutant K-Ras led to an increase of cells with both a stem cell phenotype and a mesenchymal morphology.<sup>17</sup> Moreover, the addition of the EMT promoter TGF $\beta$  accelerated the effect of oncogenic K-Ras in inducing mesenchymal morphology and stem cell phenotype in primary mammary epithelial cells.

Additional evidence links pluripotency and EMT/MET in breast cancer by showing that member factors of the core program of either contribute to the other process or even that pathways outside the core programs promote both processes. For example, a study showed that when the core pluripotency transcription factors Oct4 and Nanog were

overexpressed in stem cells sorted from the human breast cancer cell line BT-20, EMT transcription factors Snail and Slug and mesenchymal markers N-cadherin and vimentin were upregulated, while epithelial markers E-cadherin and cytokeratin 18 (CK-18) were downregulated.<sup>100</sup> The opposite effects were observed when Oct4 and Nanog were knocked down by RNA interference. Oct4 has also been associated with MET through downregulation of TGF $\beta$  signaling components TGF $\beta$ 3 and TGF $\beta$  RIII, leading to decreased Snail expression.<sup>101</sup> In cooperation with Sox2, Oct4 activates the miR-200 cluster of miRs leading to ZEB mRNA repression (Figure 3).<sup>90</sup> These data are consistent with a dual role of pluripotency factors promoting plasticity as opposed to a steady epithelial or mesenchymal state, depending on cooperations and cues from the cellular environment.

The reverse may be also true, given that EMT inducers may decrease the percentage of cells with a stem cell phenotype and suppress expression of markers associated with stemness, such as the ABC cassette transporter ABCG2 (also known as BCRP [breast cancer resistance protein]). This was shown in MCF-7 breast cancer cells which displayed decreased expression of this transporter and, as a result, a decreased percentage of side population after exposure to TGF $\beta$  and induction of EMT.<sup>102,103</sup> Similarly, in another study, neutralization of TGF $\beta$  signaling by the introduction of a dominant negative version of TGF $\beta$  RII led to a decrease of the stem cell subset.<sup>104</sup> Two additional interesting findings in the study were, firstly, that TGF $\beta$ 1 mRNA production was higher in luminal-type human breast tumors compared with



**Figure 3** An overview of the close relationship of the EMT and pluripotency networks.

**Notes:** Only some regulations are illustrated to preserve clarity. Arrows denote activation and  $\vdash$  symbols inhibition.

**Abbreviation:** EMT, epithelial–mesenchymal transition.



basal ones, a finding that agrees with the increased stem cell phenotype generally seen in basal breast cancers; and, secondly, that TGF $\beta$  downregulates Id1.<sup>104</sup> This last finding agrees with the fact mentioned in the “EMT factors and expression in breast cancer” section that a higher expression of Id proteins has been observed in basal tumors,<sup>94</sup> arguing for the need for an optimal balance of various agonists and antagonists for establishment of the required level of transcription in downstream genes that would lead to EMT or MET. In addition, these findings implicate a major EMT player, the TGF $\beta$  pathway, in the promotion of differentiation instead of stemness, at least in some settings.

Another pluripotency transcription factor that is involved in EMT is KLF4. In this case, data are controversial regarding the role of KLF4 as a breast tumor promoter or suppressor, but a clearer picture is beginning to arise. KLF4 antagonizes ZEB2 in the promoter of E-cadherin and thus upregulates this key epithelial junctional protein and promotes MET (Figure 3).<sup>105,106</sup> Moreover, there are indirect regulations of EMT involving a complex influence of KLF4 on ER and p53 signaling, both of which are regulators of EMT, as will be discussed in later sections.<sup>107,108</sup>

In addition to enhancing mammosphere formation in MCF-7 breast cancer cells, forced expression of pluripotency-promoting RNA-binding protein LIN28 promoted MCF-7 migration in vitro as well as the expression of mesenchymal marker vimentin and downregulated E-cadherin.<sup>109</sup> In contrast, knockdown of LIN28 in MDA-MB-231 breast cancer cells suppressed their migration. The pluripotency-associated miR-302 cluster is involved in MET by suppressing TGF $\beta$  RII mRNA translation, and this effect contributes to reprogramming of mouse and human fibroblasts to pluripotency.<sup>110,111</sup>

Besides the aforementioned studies of Snail and Twist, EMT-associated transcription factor FOXC2 is another example of a factor promoting both programs. FOXC2 mRNA is induced by both Snail and Twist and leads to expression of mesenchymal markers and a partial suppression of epithelial markers in breast cancer cells.<sup>112</sup> FOXC2 was found to be expressed in specimens of human breast cancers, especially the basal phenotype, in contrast to normal mammary epithelium.<sup>112</sup> In immortalized human mammary epithelial cells that have undergone EMT through forced expression of Snail, Twist, or TGF $\beta$ , FOXC2 was required for maintenance of the mesenchymal morphology.<sup>113</sup> In addition, FOXC2 expression was higher in the stem cell phenotype CD44<sup>high</sup>/CD24<sup>low</sup> mammosphere-forming fraction of breast cancer cells compared to non-stem cells with the reverse phenotype. Suppression of FOXC2 with RNA interference

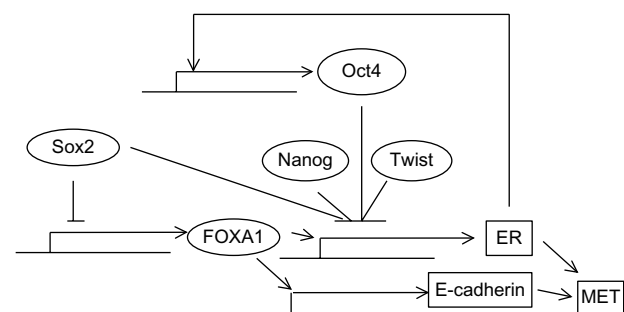
reduced the fraction of cells with the stem cell phenotype in this model.<sup>113</sup>

Overall, both types of studies, those that associate EMT with pluripotency/stem cell phenotype and those that support the involvement of specific factors in both processes, argue for a closely intertwined network that regulates both processes. A so-called metastable state in between the epithelial and mesenchymal cell conditions during EMT or MET has been described and evokes the pluripotency state whence cells retain the ability to be easily tipped toward different fates, adjusting to their environment. Moreover, EMT factors, usually associated with stemness establishment, may in certain conditions promote differentiation toward the epithelial phenotype. This is particularly true for the TGF $\beta$  cascade and may explain the well-known dual role of this signaling in carcinogenesis.

## ER and PR, cancer stem cells, and EMT in breast cancer

ER has a dual effect in breast cancer by promoting cancer initiation but being associated with less aggressive tumor biology in established cancers.<sup>114</sup> Fundamentally, ER is associated with differentiation, as seen experimentally in human ESCs expressing ER and cultured with estrogens in vitro.<sup>115</sup>

Similarly to the hierarchy of breast epithelial cells in normal breast tissue, breast cancer stem cells do not express ER irrespective of cancer subtype (whether the bulk of the tumor cells express the receptor).<sup>116,117</sup> This may be related to the fact that pluripotency factor Sox2 suppresses transcription of factor FOXA1, which is indispensable for both ER expression and function (Figure 4).<sup>118,119</sup> In addition, Sox2 suppression



**Figure 4** Regulation of ER by pluripotency and EMT factors.

**Notes:** Oct4, Sox2, Nanog, and Twist overexpression leads to suppression of ER. In addition, Sox2 has an indirect influence on ER expression and function by suppressing pioneer factor FOXA1. These effects contribute to suppression of ER expression in cancer stem cells where the pluripotency network is in place. ER promotes MET and expression of Oct4, but an established pluripotency network may render Oct4 expression independent of ER expression. Arrows denote activation and T— symbols inhibition.

**Abbreviation:** MET, mesenchymal–epithelial transition.

of FOXA1 provides a connection of pluripotency with EMT, as FOXA1 is a transcription activator of E-cadherin expression.<sup>120</sup> Another study suggested that ER signaling upregulates Oct4 through an estrogen response element in its promoter and promotes mammosphere formation in MCF-7 breast cancer cells.<sup>121</sup> This would contribute to establishment of the pluripotency network which, in turn, would suppress ER expression through suppression of FOXA1 (Figure 4). When the network is established, Oct4 expression may become independent of ER. Reciprocally, in differentiated cells that stably express ER, additional regulators may be acting to neutralize the effect of ER on Oct4 promoter in order to avert its expression that would suppress ER. The overexpression of all three pluripotency factors, Oct4, Sox2, and Nanog, leads to reduction in the expression of ER, corroborating the observation that cancer stem cells from ER-positive cancers are ER-negative.<sup>122</sup>

Although the presence of ER-negative stem cells in ER-positive cancers does not establish the cell where transformation occurs, it does imply that effects of estrogen signaling on breast cancer stem cells take place through a paracrine mode or through alternative ERs expressed by stem cells. A paracrine role of ER in breast cancer stem cell expansion through induction of FGF signaling in neighboring cells has been reported.<sup>123</sup> Breast cancer cells with acquired tamoxifen resistance, implying loss of ER expression or function or independence from this function, gain an EMT phenotype and display upregulated  $\beta$ -catenin transcription.<sup>124</sup>

Progesterone signaling is also involved in a paracrine stimulation of hormone receptor-negative cancer stem cells mediated by the RANKL–RANK ligand–receptor pair.<sup>125,126</sup> Moreover, EMT has been observed following knockdown of ER in MCF-7 breast cancer cells by RNA interference.<sup>127</sup> Upregulation of TGF $\beta$  and its receptors (TGF $\beta$  RI, II, and III), FGF1 and receptor FGFR1, EGFR, and PDGFA were observed during the transition (Figure 4). ER-negative cell lines express TGF $\beta$  RII and are able to bind the ligand in *in vitro* assays, in contrast to ER-positive breast cancer cell lines.<sup>128</sup> In addition, primary breast cancer cells with the stem cell phenotype CD44<sup>+</sup>/CD24<sup>–/low</sup> were ER-negative and TGF $\beta$  RII-positive and had a more mesenchymal phenotype than CD44<sup>+</sup>/CD24<sup>+</sup> cells that were ER-positive and did not express the TGF $\beta$  receptor.<sup>129</sup> Of note, in the same study, these latter cells seemed to be derived by the former but presented additional genetic lesions and, although they displayed no stem cell markers, they had an abnormal localization of E-cadherin, suggesting that they were not normal epithelial cells. Reciprocally, the EMT-induced Twist was shown to bind

E-boxes on the ER promoter and downregulate it through histone acetylation and DNA methylation.<sup>130</sup> Cell lines with high Twist expression were noted to be negative for ER by Western blotting, while the reverse was true for cell lines with low Twist expression. Of note, promoter methylation is a factor contributing to ER silencing in breast cancer patient specimens.<sup>131</sup>

The intracellular environment, in the form of parallel pathways being present and functional, is clearly also of importance in the response to ER signaling. The hedgehog (HH) pathway, for example, may be activated by ER and contributes to both cancer stem cell development and EMT.<sup>132</sup> The Notch signaling pathway is also activated by ER in MCF-7 breast cancer cells.<sup>133</sup> Both Notch receptor and its ligand Jagged1 are upregulated after estradiol treatment of these cells. Given the well-known role of Notch signaling in pluripotency<sup>134</sup> and EMT,<sup>73</sup> its activation by ER would be expected to promote both, although this was not specifically investigated in the study.<sup>133</sup> Polycomb complex member protein BMI1 is additionally a target of ER stimulation by estrogens and promotes stemness and EMT.<sup>135</sup>

Overall, ER signaling appears to have multiple effects in hormone receptor-positive breast cancer. In non-stem cells expressing the receptor, it acts inside the cell to suppress EMT and safeguard epithelial phenotype, but if parallel pathways such as HH and Notch are in place, it may promote stemness and EMT. In the small population of ER-negative stem cells, ER acts, through intermediate effectors, to promote their proliferation and expansion in a paracrine manner. As a result, inhibition of ER by anti-hormonal treatments may actually counterintuitively promote EMT in the bulk breast cancer cell population (except if parallel pathways are operational). These cells would be less able to metastasize because they lack the plasticity of the pluripotency network. In contrast, ER inhibition would tend to inhibit EMT in adjacent stem cells that have the pluripotency network in place and would be able to metastasize if EMT is induced in them. The implications of these considerations in ER-positive breast cancer therapeutics would lie in attempts to delay or reverse hormonal treatment resistance by inhibition of paracrine pathways, but also in attempts to dissect and prevent any EMT-promoting effect that hormone inhibition would have in the bulk tumor cells.

## Tumor suppressor p53 as a major suppressor of pluripotency and EMT

p53, a well-known tumor suppressor and the most commonly mutated gene in cancers, is involved in inhibition

of tumor development by promoting processes such as cell cycle arrest, apoptosis, and DNA damage repair. In addition, p53 regulates both EMT and pluripotency, being an inhibitor of EMT and a major block to pluripotency. p53 is a direct or indirect suppressor of the expression of pluripotency factors, an example of the former being direct suppression of Nanog and of the latter induction of miR-145 which suppresses Oct4 and Sox2.<sup>136</sup> p53 is a transcriptional repressor of CD44 which is a transmembrane receptor protein and a hallmark of breast cancer stem cells.<sup>137</sup> p53 is also a suppressor of EMT by inducing miRs of the miR-200 family that are suppressors of EMT transcription factors and also ligase HDM2 that ubiquitinates Slug leading to its degradation by the proteasome.<sup>138</sup> In contrast, mutant p53 may promote EMT. In MCF-7 and MCF-10 cells, introduction of mutant p53 decreased the expression of E-cadherin and epithelial CK-18, promoted EMT, and altered cell polarity.<sup>139,140</sup> In addition, mutant p53 leads to activation of transcription factor NF- $\kappa$ B, a prosurvival factor.<sup>141</sup> A role of NF- $\kappa$ B activation is proposed in stem cells of ER-positive breast cancers. These stem cells, despite being hormone receptor-negative, may be stimulated by hormone receptor-positive cancer cells constituting the bulk of the tumor in a paracrine manner, as discussed also in the “ER and PR, cancer stem cells, and EMT in breast cancer” section.<sup>142</sup> Hormone receptor-positive cells are stimulated by PR (an ER target gene) signaling to upregulate and secrete receptor activator of NF- $\kappa$ B ligand (RANKL), which then ligates its receptor RANK in adjacent hormone receptor-negative stem cells activating intracellular pathways of survival, among which the NF- $\kappa$ B pathway is a prominent one.<sup>143</sup> Moreover, NF- $\kappa$ B promotes stem cell expansion, and its inhibition by I- $\kappa$ B inhibitor of NF- $\kappa$ B in mammary epithelial cells in transgenic mice delayed tumor development and suppressed tumor stem cell expansion.<sup>143</sup> A role of NF- $\kappa$ B in EMT induction in mammary cancer cells is mediated through upregulation of transcription regulators Snail and Twist.<sup>144</sup> Thus, mutant p53 may cooperate, through NF- $\kappa$ B activation, with hormone receptors in activation of survival pathways or substitute for the function of hormone receptors in hormone receptor-negative cancers.

In breast cancers, mutations of p53 are present with varying frequencies depending on the subtype. In luminal A carcinomas, an incidence of 17% was found, while in luminal B carcinomas, this incidence is 41% and, in ER-negative cancers, it is 50% in Her2-positive carcinomas and 88% in triple-negative.<sup>145</sup> Paradoxically, p53 mutations have been associated with responses to doxorubicin/cyclophosphamide chemotherapy in contrast to patients with wild-type p53, in

whom no complete responses were obtained with the same regimen. This may be explained by the program (cell cycle arrest versus apoptosis) that is executed following p53 activation in breast cancer cells. Cellular survival and senescence was observed in wild-type p53 breast cancers, while aberrant mitoses followed by apoptosis, possibly mediated by alternative non-p53-dependent pathways, was observed in p53-mutant breast cancers.<sup>145</sup> The program activated by wild-type p53 is defined in part by various posttranslational modifications that modify its affinity for different promoters of target genes.<sup>146</sup> An important modification favoring apoptosis over cell cycle arrest is phosphorylation at serine in position 46, which is mediated by kinases HIPK2 and DYRK2. Thus, availability and activity or lack thereof of these kinases may modulate the outcome of p53 activation in cancer cells. In addition to promoting p53-induced apoptotic programs, DYRK2 phosphorylates Snail in a serine residue at position 104 leading to a second phosphorylation by kinase GSK3 $\beta$  followed by ubiquitination by ligase  $\beta$ TrCP and degradation by the proteasome. As a result, downregulation of DYRK2 observed in breast cancers leads to Snail accumulation and EMT, promoting the invasive potential of these cancers.<sup>147</sup> At the same time, it may contribute to treatment resistance by favoring the cell cycle arrest program of p53 instead of apoptosis.

## Pluripotency and EMT factors and prognosis of breast cancer

Several studies have examined the prognostic value of the expression of pluripotency and EMT factors in human breast cancer.

In a series of 90 breast cancer patients, Oct4 expression positivity, defined as more than 10% of tumor cells expressing the protein in their nucleus, was associated with decreased disease-free (DFS) and overall (OS) survival in Cox univariate analysis, although it lost its statistical significance in multivariate analysis, possibly due to its significant association with established prognostic parameters such as lymph node positivity and grade.<sup>148</sup> In another series, with 126 breast cancer patients across subtypes, using less stringent criteria of 1% of cells as the cutoff for positivity, both Oct4 and Nanog expression and their combination were associated with reduced OS.<sup>100</sup>

Copy number gain of chromosome 3q, where the *Sox2* gene is located, is a predictor of recurrence in breast cancer patients, and elevated Sox2 expression is associated with tamoxifen resistance.<sup>149</sup> Another study found Sox2 mRNA expression to be associated with reduced DFS but not OS in

breast cancer patients.<sup>150</sup> mRNA expression was correlated with protein expression by immunohistochemistry in all samples tested. In contrast to Sox2, levels of Nanog or KLF4 mRNA were not predictive of outcome in this study.<sup>150</sup> An extensive study of over 500 breast cancers also confirmed the association of Sox2 expression by immunohistochemistry with poor DFS.<sup>151</sup> In addition, Sox2 was negatively associated with ER and PR status and more commonly seen in metastatic lymph nodes than in the corresponding primary tumor site.

A correlation with poor prognosis was suggested for Nanog in a microarray study, while no prognostic significance was attributed to Oct4, Sox2, or c-myc in this study.<sup>152</sup> This study examined protein expression, a fact that may explain the contradictory results with the previously mentioned study that examined mRNA.<sup>150</sup>

KLF4 expression was also examined in the above study and was determined to be, in contrast to Nanog expression, a marker of good prognosis.<sup>152</sup> In contrast, another study maintained that, in early breast cancers (stage I and IIA), increased nuclear accumulation of KLF4, as determined by immunohistochemistry, was associated with a statistically significant worse prognosis, an effect that was not observed in more advanced stages.<sup>153</sup>

c-myc expression was examined by immunohistochemistry in a breast cancer series of 206 patients and was shown to have little independent value as a prognostic marker compared with established markers.<sup>154</sup> Nevertheless, cytoplasmic expression of c-myc in central areas of tumors was associated with cases being already metastatic at diagnosis, implying aggressiveness. Interestingly, in this study, nuclear expression of c-myc was inversely correlated with ER expression.<sup>154</sup> Another analysis of Her2-positive patients confirmed that higher (>30%) nuclear staining for c-myc had no prognostic significance and these patients had similar outcomes compared to Her2-positive patients with lower levels of c-myc nuclear staining and benefited equally by the addition of trastuzumab to their treatment.<sup>155</sup> In contrast to these data, in patients with sporadic breast cancers and BRCA1 gene functional insufficiency due to promoter methylation or downregulation at the protein level, c-myc overexpression was associated with adverse clinical outcomes.<sup>156</sup> Two other studies examined c-myc gene amplification and its prognostic significance in breast cancer. An investigation in a series of 181 node-negative, mostly (two-thirds) small (T1), ER-positive, postmenopausal breast cancers showed, using differential polymerase chain reaction, that c-myc was amplified in approximately 20% of patients and the amplified cases had a worse DFS but no difference in OS compared

with non-amplified counterparts.<sup>157</sup> The other study, using CISH, found a lower percentage of c-myc amplification of 5%–10% across breast cancer subtypes, and this amplification predicted worse OS.<sup>158</sup>

LIN28 overexpression in breast cancer cells leads to Her2 upregulation and has been correlated with poor prognosis in breast cancer patients.<sup>159</sup> In addition, LIN28B was associated with aggressive clinicopathologic characteristics, such as lymph node positivity and expression of the proliferation marker Ki67, in a series of 190 breast cancer patients.<sup>160</sup>

An association of higher expression of EMT factors with a shorter relapse-free period was shown specifically in a study of ER-positive patients.<sup>93</sup> High coexpression of Snail and Twist could identify patients with worse prognosis in this tissue microarray study. In a more recent extensive tissue microarray study of 1,043 breast cancer patients, nuclear Snail expression correlated with known clinicopathologic prognostic factors such as stage, grade, lymph node status, and ER expression.<sup>88</sup> High Snail expression was an adverse prognostic marker for OS in luminal B, Her2-positive, and basal-like cancers, but not in the luminal A subtype.<sup>88</sup> Another study that used previously published microarray data confirmed that higher Snail mRNA expression was associated with decreased 5-year relapse-free survival in breast cancer patients.<sup>161</sup>

Slug in cooperation with Sox9 (a major factor mediating sexual development) has been found to convert mammary epithelial cells to stem cells with increased ability of mammosphere formation and tumor development in mice.<sup>162</sup> The two factors, Slug and Sox9, when coexpressed in breast cancer patients, conferred decreased survival compared with patients expressing only one or none of the two factors.<sup>162</sup>

High transcription factor Twist1 mRNA expression was associated with decreased DFS and OS in patients with ER-positive lymph node-negative breast cancer.<sup>163</sup> Another report showed that both high Twist and high Slug mRNA expression were directly associated with poor outcomes.<sup>87</sup> In contrast to previously discussed data, higher Snail expression was associated with better outcomes in this study.

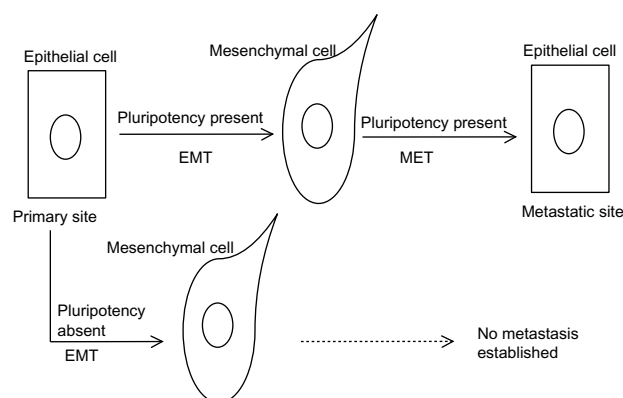
Discordant data should be viewed with the perspective of tumor heterogeneity in mind. As the cancer stem cell theory states, not all cancer cells in the bulk or different areas of a given tumor have the same gene expression and tumor propagation potential at a given time. To ascertain this, a study comparing Snail, Slug, and Twist expression from primary breast cancers and lymph node metastases showed that expression of these genes correlated with OS only when studied in the lymph nodes but not in the primaries.<sup>164</sup>



In addition, patients that displayed a conversion of Snail from negative in the primary tumors to positive in their lymph node metastases had a significantly worse DFS and OS compared to patients that did not convert.<sup>164</sup>

## Integration of pluripotency and EMT programs in breast cancer prognosis and therapy

Although, as discussed above, the close relationship of EMT and pluripotency programs has been revealed in cancer in general and in breast cancer in particular,<sup>16,17,165</sup> the molecular connections of pluripotency/plasticity and EMT/MET transitions at the level of their respective core transcription factors and epigenetic modifications have only started to be specifically addressed. The pluripotency network establishes bispecific chromatin methylations on important developmental genes that keep them suppressed but retain them in a primed state ready for future expression or for more permanent suppression upon receiving additional input. The EMT core network may act on this environment to skew the balance toward EMT, and later on in the metastatic site back toward MET, taking advantage of the plasticity conferred by the established pluripotency network on cancer stem cells.<sup>166</sup> This model implies that the EMT network will work together with the pluripotency network to endow cancer stem cells with metastatic potential (a hallmark of cancer), while in cells with no pluripotency network in place, the effect would not be the establishment of metastatic disease because these cells would lack the required plasticity and would be unable to undergo a MET (Figure 5). This requirement also predicts that



**Figure 5** A proposed model of the cooperation of pluripotency and EMT/MET processes in metastasis establishment.

**Notes:** According to this model, a cell undergoing EMT would be able to establish a metastatic focus only if the pluripotency network is in place to endow it with the genetic plasticity required to undergo the reverse process, MET.

**Abbreviations:** EMT, epithelial–mesenchymal transition; MET, mesenchymal–epithelial transition.

the strongest oncogenic events would promote both pathways in parallel. An additional observation supporting the close interrelationship of the two networks comes from examination of promoter sequences upstream of transcription start sites of genes encoding for the human transcription factors of the pluripotency and EMT networks. These preliminary results show that all promoter sequences possess multiple putative binding sites for other transcription factors of the two networks (Voutsadakis, unpublished data, 2015). The importance of both programs for metastatic dissemination has recently been investigated in circulating tumor cells which have been shown to coexpress markers of both processes.<sup>167</sup> The fact that such cells are circulating in the blood of cancer patients is an initial proof of the pathophysiologic importance of the two programs acting in concert and provides opportunities for both prognostication and treatment of breast cancer and other cancers.

Given the multiple factors that are needed to act in a coordinated manner in order to establish the pluripotency and EMT networks, it may be necessary to examine several of them in a multiplex platform similar to the ones currently in clinical use to derive the full prognostic value of the presence of the two networks in breast cancer cells. In this type of prognostic panel building, already published genomic sets with clinical annotation could be of significant help. For example, a search of such a set of breast cancer patients using the online tool survExpress (<http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp>)<sup>168</sup> shows that a panel of transcription factors from the pluripotency and EMT programs performs quite similarly in prediction of OS to the panel of genes included in the Oncotype Dx test when tested in a set of breast cancers published by The Cancer Genome Atlas (Voutsadakis, unpublished data, 2015). Further refinements of such panels may guide future developments of even better prognostic tools for validation and use in the clinic.

## Disclosure

The author reports no conflicts of interest in this work.

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