Identifying and targeting tumor-initiating cells in the treatment of breast cancer

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Abstract

Breast cancer is the most common cancer in women (excluding skin cancer), and it is the second leading cause of cancer-related deaths. Although conventional and targeted therapies have improved survival rates, there are still considerable challenges in treating breast cancer, including treatment resistance, disease recurrence, and metastasis. Treatment resistance can be either de novo – because of traits that tumor cells possess before treatment – or acquired – because of traits that tumor cells gain in response to treatment. A recently proposed mechanism of de novo resistance invokes the existence of a specialized subset of cancer cells defined as tumor-initiating cells (TICs), or cancer stem cells (CSCs). TICs have the capacity to self-renew and to generate new tumors that consist entirely of clonally derived cell types present in the parental tumor. There are data to suggest that TICs are resistant to many conventional cancer therapies and that they can survive treatment in spite of dramatic shrinkage of the tumor. Residual TICs can then eventually regrow, which results in disease relapse. It has also been hypothesized that TIC may be responsible for metastatic disease. If these hypotheses are correct, targeting TICs may be imperative for achieving a cure.

In the present review, we discuss evidence for breast TICs and their apparent resistance to conventional chemotherapy and radiotherapy as well as to various targeted therapies. We also address the potential impact of breast TIC plasticity and metastatic potential on therapeutic strategies. Finally, we describe several genes and signaling pathways that appear to be important for TIC function and may represent promising therapeutic targets.

Introduction

In recent years, breast cancer patients, clinicians, and scientists have rightfully celebrated small, but significant, improvements in breast cancer outcome. These improvements have been attributed to better methods for early detection, enhanced screening efforts, and the availability of more effective targeted therapeutics for the treatment of the two largest clinically defined subtypes of breast cancers – those that express the estrogen receptor (ER\(^+\)) (with or without co-expression of the progesterone receptor (PR)) and those with overexpression or amplification of the human epidermal growth factor 2 (Erbb2) gene (a.k.a. HER2\(^+\)). These two subtypes of breast cancer account for ~70 and 15–20% of all cases respectively. The remaining subtype, triple-negative breast cancer (TNBC), lacks expression of ER, PR, and HER2. To date, there are no targeted agents to combat TNBC, and its prognosis remains poor.
Despite recent progress, several major clinical problems remain. Chief among these are the issues of treatment resistance, disease recurrence, and metastasis. For example, whereas many ER$^+$ tumors respond to ER-targeted therapies (antiestrogens such as tamoxifen and aromatase inhibitors such as anastrozole, letrozole, and exemestane), de novo and acquired resistance is common (Burstein et al. 2014). Similarly, recent clinical trials have shown that up to 64% of HER2$^+$ patients can show pathological complete response to combination treatment with dual anti-HER2 targeted therapy (Gianni et al. 2012, Schneeweiss et al. 2013, de Azambuja et al. 2014, Cortazar et al. 2014). However, a significant percentage of patients are resistant to these agents. In TNBC, treatment generally involves using multiagent chemotherapy along with surgery. Unfortunately, not all patients who receive chemotherapy show clinical benefit, and side effects can be significant. In the case of disease recurrence, the recurrent cancer can be refractory to the original treatment.

Breast cancer has long been recognized as a heterogeneous disease, and this heterogeneity has been invoked to explain, at least in part, differences in treatment response, recurrence potential, and metastatic behavior. Tumor heterogeneity exists at the histological and molecular levels within a single tumor (intratumoral) and between different tumors (inter tumoral). Recent gene expression profiling is beginning to reveal the full extent of inter tumoral heterogeneity. For example, independent of the three clinically defined subtypes of breast tumors, at least six molecular subtypes of breast cancer have been identified: luminal A, luminal B, HER2-enriched, normal-like, basal-like, and claudin-low (Perou et al. 2000, Herschkowitz et al. 2007). The luminal subtypes are generally ER$^+$. The HER2-enriched subtypes are typically ErbB2$^+$ and are also generally ER$^-$. Tumors in the basal-like subtype are generally triple-negative. To date, ~60–70% of identified claudin-low tumors have been triple-negative (Prat & Perou 2011). More recently, TNBC have been evaluated in large numbers and show at least six subclasses (Lehmann et al. 2011).

Although it has been less well studied than intratumoral heterogeneity, breast tumors also show intratumoral heterogeneity. As in the normal mammary gland, where cellular heterogeneity has been recognized and studied for decades, phenotypic heterogeneity at the cellular level is also common within breast tumors. For example, in the normal mammary gland, only 30–40% of cells express ER and PR; likewise, in ER$^+$ breast tumors, ER$^+$ cells express variable levels of ER protein, and up to 99% of all tumor cells may not express any detectable ER at all (Harvey et al. 1999, Hammond et al. 2011). In a similar fashion, PR is not generally expressed in every cell in PR$^+$ tumors. Although it is not currently useful in clinical decision making, the expression of many other protein markers (e.g., cytokeratin 5, CD44, CD24, PTCH1, and SMO) is also known to vary from cell to cell in some breast cancers (Abd El-Rehim et al. 2004, Moraes et al. 2007, Marotta et al. 2011). In addition to simple variability at the protein expression level, there is now known to be genetic heterogeneity within tumors. For instance, single-cell sequencing data demonstrate that there are extensive clonal diversities within a single tumor as a result of the low-frequency point mutations that evolved during tumor development. Noticeably, the mutation frequency is 13 times higher in TNBCs than it is in luminal-type tumors, which suggests an increase in heterogeneity in TNBC (Wang et al. 2014a).

It stands to reason that the observed phenotypic and genetic heterogeneity within a given tumor likely results in functional heterogeneity. Most importantly from a clinical perspective, these heterogeneous tumor cell subpopulations may show different responses to therapy, different division potentials, and varied metastatic properties. Therefore, a better understanding of the mechanisms that contribute to intratumoral heterogeneity in breast tumors, particularly regarding the tumor-initiating subpopulations, is critical for improving current treatment options.

**Heterogeneity and the cancer stem cell hypothesis**

The cancer stem cell (CSC) hypothesis was developed in part to explain the intratumoral heterogeneity. According to this hypothesis, many cancers have a unique subset of cells that are referred to as CSCs and have the capacity to self-renew and give rise to other cancer cell types, which creates a hierarchically organized tumor (Visvader & Lindeman 2012). Furthermore, these CSCs are thought to be the main drivers of tumor growth. Evidence also indicates that CSCs are more resistant to conventional therapies, which suggests that they also play an important role in mediating tumor relapse (Creighton et al. 2009). Therefore, this hypothesis provides a plausible explanation for different types of treatment failure, although the mechanisms of resistance and the proportion of CSCs may vary between different tumors. We and many other groups prefer the term ‘tumor-initiating cells (TICs)’ instead of CSCs to distinguish.
these cells from normal stem cells and to emphasize their tumor-initiating capacity.

The identification of TICs in breast tumors

Putative breast TIC subpopulations can be isolated by fluorescence-activated cell sorting (FACS) using several combinations of cell surface and non-cell surface markers (Table 1), followed by functional assays that demonstrate their enriched tumorigenic potential. For example, subpopulations of human breast tumor cells have been shown by limiting dilution transplantation (LDT) assays to have enriched tumorigenic potential (Al-Hajj et al. 2003, Ginestier et al. 2007). In addition, serial transplantation assays have been performed to demonstrate the self-renewal capacity of breast TICs, and the heterogeneity of the regenerated daughter tumors has provided evidence of their differentiation capacity (Al-Hajj et al. 2003). Mammosphere formation efficiency (MSFE) assays have also been developed as in vitro surrogates for LDT assays to demonstrate the self-renewal capacity of breast TICs (Dontu et al. 2003). However, MSFE and regenerative cell frequency may not necessarily correlate in all cases, which may limit the interpretation of in vitro assays (Moraes et al. 2007).

To isolate TICs from breast tumors, researchers frequently use fluorescent-conjugated antibodies to cell surface markers, which are specifically expressed or enriched in TIC populations, combined with FACS.

Al-Hajj et al. (2003) published the first markers to enrich for human breast TICs in 2003. The authors used two cell surface markers, the glycoproteins CD44 and CD24, to isolate CD44+/CD24−/low/Lin− cells from primary human/xenograft breast tumors, and they showed that this cell population is more tumorigenic than the other cell populations, which suggests that it is enriched for TICs. Importantly, this tumor-initiating advantage is maintained during serial passages, and the new generations of tumors contain phenotypically diverse populations (Al-Hajj et al. 2003). Interestingly, CD44 and CD24 markers fail to enrich for TICs in several murine xenograft models that are ER− and triple-negative (Meyer et al. 2010), which suggests that TIC populations may vary between different breast cancer subtypes.

Surrogate markers for CD44+/CD24− cells have also been suggested, including ganglioside GD2 (a glycosphingolipid) and protein C receptor (PROCR) (Shiptsin et al. 2007, Battula et al. 2012). Almost all GD2+ cells are CD44+/CD24−, and knockdown of GD3 synthase, an enzyme involved in the synthesis of GD2, in GD2+ cells reduces their TIC properties, which suggests that GD2 and CD44 may be phenotypic indications of underlying mechanisms that drive TIC function. PROCR is a cell surface receptor that is specifically expressed in CD44+ cells. Unlike CD44, which is also expressed in leukocytes and myofibroblasts, PROCR expression is restricted to CD44+ epithelial cells. PROCR was initially identified as a cell surface receptor that is specifically expressed in CD44+ cells.

Table 1  Functionally defined TIC markers for human breast tumors and mouse mammary tumors

<table>
<thead>
<tr>
<th>TIC markers</th>
<th>Tumor subtypes</th>
<th>TIC frequency before enrichment</th>
<th>TIC frequency after enrichment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human breast TIC markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44+/CD24low</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Al-Hajj et al. (2003)</td>
</tr>
<tr>
<td>ALDH+</td>
<td></td>
<td>Basal-like</td>
<td>NR</td>
<td>Ginestier et al. (2007)</td>
</tr>
<tr>
<td>STAT3–GFP</td>
<td></td>
<td>Claudin-low (cell line xenografts)</td>
<td>NR</td>
<td>Liu et al. (2014)</td>
</tr>
<tr>
<td>EpCAM+/CD49fhi</td>
<td></td>
<td>Triple-negative</td>
<td>1/71</td>
<td>Wei et al. (2014)</td>
</tr>
<tr>
<td>CD44+/CD49fhi/CD1332hi</td>
<td></td>
<td>ER</td>
<td>NR</td>
<td>Lee et al. (2014)</td>
</tr>
<tr>
<td>Mouse mammary TIC markers</td>
<td></td>
<td></td>
<td></td>
<td>Meyer et al. (2010)</td>
</tr>
<tr>
<td>Sca1+/CD24+</td>
<td></td>
<td>Luminal (MMTV-Neu)</td>
<td>1/303</td>
<td></td>
</tr>
<tr>
<td>CD49f+/CD61hi</td>
<td></td>
<td>Luminal (MMTV-Neu)</td>
<td>1/70</td>
<td></td>
</tr>
<tr>
<td>CD24+/CD49hi</td>
<td></td>
<td>Luminal (MMTV-PyMT)</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>CD29hi/CD24−/CD61+</td>
<td></td>
<td>Basal-like (MMTV-Wnt1)</td>
<td>1/303</td>
<td></td>
</tr>
<tr>
<td>Thy1+/CD24+</td>
<td></td>
<td>Basal-like (MMTV-Wnt1)</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>EpCAMlow/CD49hi</td>
<td></td>
<td>Basal-like (MMTV-Wnt1)</td>
<td>1/79</td>
<td></td>
</tr>
<tr>
<td>CD24hi/CD49fhi</td>
<td></td>
<td>Basal-like (MMTV-Wnt1)</td>
<td>1/71</td>
<td></td>
</tr>
<tr>
<td>CD29hi/CD24+</td>
<td></td>
<td>Basal-like (p53-null)</td>
<td>1/21 583</td>
<td></td>
</tr>
<tr>
<td>TOP–GFP</td>
<td></td>
<td>Basal-like (p53-null)</td>
<td>1/148–1/225</td>
<td></td>
</tr>
<tr>
<td>CD24+/CD29+</td>
<td></td>
<td>Basal-like (Brca1-deficient)</td>
<td>NR</td>
<td></td>
</tr>
</tbody>
</table>

NR, not reported.
an embryonic stem cell (ESC) marker (Ramalho-Santos et al. 2002), which suggests that it may play a general role in stem cell function.

More recently, integrins have been identified as human breast TIC markers, because they are differentially expressed between breast TICs and non-TICs. For example, the α6 integrin (CD49f) marks TICs in ER− and triple-negative breast tumor xenografts (Meyer et al. 2010, Lee et al. 2014). Using a combination of CD49f, CD44, and CD24 may further enrich for breast TICs from patient breast tumor samples, as compared to CD44 and CD24 alone; MSFE assays show that the self-renewal capacity is predominantly in the CD44high/CD24low/CD49f+ population but not in the CD44high/CD24low/CD49f− population (Ghebeh et al. 2013).

A different set of cell surface proteins have been used to enrich for TICs from mouse mammary tumors. In mouse mammary tumor virus (MMTV)-Neu tumors, the stem cell antigen 1 (Sca1)+/CD24+ combination and the β3 integrin CD61 combined with CD49f (CD49fhi/CD61hi) enrich for TICs (Liu et al. 2007, Lo et al. 2012). In MMTV-PyMT tumors, the CD24+/CD49f+ combination enriches for TICs (Ma et al. 2012). In MMTV-Wnt1 tumors, the CD61+/CD29hi/CD24+ combination, the thymocyte antigen 1 (Thy1)+/CD24+ combination, the EpCAMlow/CD49fhi combination, and the CD24hi/CD49fhi combination all enrich for TICs (Cho et al. 2008, Vaillant et al. 2008, Feng et al. 2014, Lee et al. 2014). In both p53-null and Brca1-deficient tumors, TICs are enriched in the β1 integrin (CD29)hi/CD24+ cells (Vassilopoulos et al. 2008, Zhang et al. 2008).

Non-cell surface markers have also been used successfully to enrich for TICs. For example, aldehyde dehydrogenase (ALDH) activity has been used to identify stem cells in mouse mammary glands and human breast tumors (Ginestier et al. 2007). Results showed that ALDH+ cells perform better than negative cells do in LDT assays, and they generate tumors that recapitulate the heterogeneity of the parental tumors in terms of enzyme activity. However, a different study found that although many basal/mesenchymal breast cancer cell lines are positive for ALDH activity, the majority of luminal-type breast cancer cell lines are negative (Charafe-Jauffret et al. 2009), which suggests that distinct breast TIC populations may exist in different types of breast tumors. That study also revealed the limited overlap between the CD44+/CD24− and ALDH1+ populations, which suggests that there may be distinct breast TIC populations even within individual tumors. Therefore, similar to the restricted application of CD44+/CD24−/flow/Lin− markers, ALDH activity may only be useful for certain breast tumor subtypes.

Because several cellular signaling pathways are key regulators of breast TIC function, fluorescent reporters of their downstream activity can also serve as functional breast TIC markers. For example, a recent study showed that a Wnt signaling reporter is preferentially activated in TICs in basal-like, p53-null mouse mammary tumors and that cell populations that are isolated based on the expression of the reporter are highly enriched for TICs (Zhang et al. 2010). The utility of this reporter has not been explored fully in models of human breast cancer.

More recently, a lentiviral STAT3–EGFP reporter was shown by both MSFE and limiting-dilution transplantation to be a potent and functionally relevant breast TIC marker in claudin-low cell line xenograft models of human breast cancer (Wei et al. 2014). These fluorescent pathway reporters facilitate the visualization of specific signaling pathway activity in live cells and, more broadly, facilitate stem cell research by enabling direct FACS.

It is worth noting that there are huge variations between TIC frequencies estimated by different TIC markers. For example, breast cancer cell line MDA231 contains 85% CD44+/CD24− cells vs 0.88% ALDEFLUOR+ cells, whereas breast cancer cell line SKBR3 contains 0% CD44+/CD24− cells vs 95.3% ALDEFLUOR+ cells (Sheridan et al. 2006, Charafe-Jauffret et al. 2009). Table 2 summarizes the percentage of marker-expressing cells in frequently used breast cancer cell lines.

Researchers have tried to consolidate the discrepancy by applying different TIC markers to different molecular subtypes of breast cancers (Ricardo et al. 2011) as well as by arguing that different sets of markers represent TICs in different states of mesenchymal–epithelial transition (MET)/epithelial–mesenchymal transition (EMT) (Liu et al. 2014). Unfortunately, even within the same molecular subtypes and using the same TIC marker set, marker expression does not correlate with TIC frequency estimated by functional assays. For example, the claudin-low-type human breast cancer cell lines SUM159 and MDA231 contain comparable percentages of CD44+/CD24− cells (95% vs 98%; Fillmore & Kuperwasser 2008), yet SUM159 exhibits an MSFE that is much higher than that of MDA231, whereas MDA231 display a much higher tumorigenic capacity than that of SUM159 as judged by LDT assays (Wei et al. 2014). Similarly, although the percentages of CD44+/CD24−/ESA− cells in SUM149 and SUM159 correlate well with their MSFEs (Fillmore & Kuperwasser 2008), they fail to predict the relative tumorigenic capacity of SUM159 and MDA231 cells (Wei et al. 2014).
Furthermore, the lack of a correlation between MSFEs and tumorigenic capacity has been widely observed. For example, LDT assay has shown that the breast cancer cell line SUM149 has a higher MSFE after normalization to proliferation but a lower tumor-formation frequency as compared to SUM159 (Fillmore & Kuperwasser 2008). A similar but more dramatic result has been observed between SUM159 and MDA231 xenograft cells (Wei et al. 2014). One recent study also showed that a marker that bears cells in different cell lines responds to therapies inconsistently, which highlights a lack of correlation between TIC markers and treatment responses (Liu et al. 2014).

To date, none of the identified breast TIC markers has been shown to be universal. Thus, continuing to search for novel markers and using novel combinations of current markers will presumably improve TIC identification and isolation and may lead to a better understanding of breast TICs. Because of the inconsistent interpretation of TIC markers within and across breast cancer samples, it is important to rigorously evaluate TIC frequency by LDT assays whenever possible rather than simply relying on TIC markers.

### Breast TICs may be resistant to conventional systemic therapies

The content of TICs in breast tumors has been shown to be closely related to clinical outcome. For example, most triple-negative tumors belong to either a basal-like or a claudin-low subtype (Herschkowitz et al. 2012). Claudin-low tumors have worse prognoses as compared to luminal A tumors as well as clear enrichment for TIC-associated signatures (Prat et al. 2010, Herschkowitz et al. 2012). The aggressiveness of primary breast cancers has also been associated with TIC frequency: poorly differentiated tumors display a higher content of TICs in xenotransplantation experiments (Pece et al. 2010, Usary et al. 2013). Consistent with these findings, accumulating evidence has revealed that breast TICs are resistant to diverse types of breast cancer therapies.

### Resistance to chemotherapy

Eventual relapse following chemotherapy has been a major challenge in treating breast cancer (1998). The existence of de novo chemoresistant breast TICs could be a major cause of disease relapse. There are two essential pieces of evidence that support this idea: i) chemotherapy treatment enriches for cells that express markers of breast cancer TICs. ii) Tumors enriched in markers of breast TICs are comparatively resistant to chemotherapy treatment. For the former piece of evidence, it has been shown that, following chemotherapy, residual breast cancers and cancer cell lines have increased CD44^{high}/CD24^{low} subpopulations, increased MSFE and tumor-initiating efficiency, and enriched expression of TIC signature genes (Yu et al. 2007, Fillmore & Kuperwasser 2008, Li et al. 2008, Creighton et al. 2009). For the latter, it has been shown that breast tumors that have been classified as claudin-low,

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Percentage of CD44^{+}/CD24^{−}</th>
<th>Percentage of CD44^{+}/CD24^{−}/ESA^{−}</th>
<th>Percentage of ALDH^{+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT474</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HCC1937</td>
<td>57</td>
<td>2.26</td>
<td></td>
</tr>
<tr>
<td>H5758T</td>
<td>64</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>MCF10A</td>
<td>16</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>MCF12A</td>
<td>5</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>MCF7</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>85</td>
<td>1.8</td>
<td>2.65</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>72</td>
<td>1.8</td>
<td>0.2</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>0</td>
<td>0</td>
<td>3.54</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SKBR3</td>
<td>0</td>
<td>95.3</td>
<td></td>
</tr>
<tr>
<td>SUM1315</td>
<td>97</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>SUM149</td>
<td>92</td>
<td>5</td>
<td>5.96</td>
</tr>
<tr>
<td>SUM159</td>
<td>55</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>SUM225</td>
<td>55</td>
<td>1</td>
<td>5.49</td>
</tr>
<tr>
<td>T47D</td>
<td>0</td>
<td>0</td>
<td>2.12</td>
</tr>
<tr>
<td>ZR75.1</td>
<td>0</td>
<td>0</td>
<td>1.02</td>
</tr>
</tbody>
</table>


*Percentage estimated from bar graph.*
a molecular subtype that is enriched for TIC features, are relatively resistant to chemotherapy (Usary et al. 2013).

The hypothesized quiescent nature of breast TICs may confer their chemoresistance, because efficient induction of apoptosis by chemotherapeutic agents usually requires cell division (Naumov et al. 2003, Moore & Lyle 2011). There are several lines of evidence showing that breast TICs are slow cycling: i) markers from the gene expression signature that is derived from quiescent normal human mammary stem cells can be used to isolate breast TICs, which suggests that breast TICs are correspondingly quiescent (Pece et al. 2010). ii) The dye retention side population (SP) is enriched for TICs in breast cancer cell lines, and it shows increased expression of negative cell cycle regulators (Goodell et al. 1996, Hirschmann-Jax et al. 2004, Patrawala et al. 2005, Zhou et al. 2007). iii) CD44+/CD24−/low cells that have been isolated from a panel of breast cancer cell lines are slow cycling and resistant to chemotherapies (Fillmore & Kuperwasser 2008). Hence, although it may not be true in all tumors, the slow-cycling nature of breast TICs may serve as a potential mechanism for chemoresistance (Moore & Lyle 2011).

Another potential mechanism that may confer chemoresistance to breast TICs is the increased presence of ATP binding cassette (ABC) transporters, which actively pump drugs out of cells, relative to the bulk of the tumor. The binding cassette (ABC) transporters, which actively pump drugs out of cells, relative to the bulk of the tumor. The breast cancer resistance protein (BCRP) was first identified in a multidrug resistant (MDR) subline of MCF7, and it is involved in in vitro MDR (Doyle et al. 1998). Likewise, the increased expression of the P-glycoprotein (Pgp), another well-characterized ABC transporter, is associated with doxorubicin resistance in multiple breast cancer cell lines (Turton et al. 2001). In patient breast tumor samples, high BCRP expression is correlated with high HER2 expression, lymph node metastasis, and an advanced stage of breast cancer (Xiang et al. 2011). Pgp shows higher expression after treatment with chemotherapy, which suggests that tumor cells that express Pgp may resist to chemotherapy (Rudas et al. 2003). Furthermore, there is evidence to suggest that BCRP and Pgp are preferentially expressed in breast TICs. For example, the SP cells of MCF7 cells, which is enriched for TICs, expresses higher levels of ABC transporters than non-SP cells do (Zhou et al. 2007). On the other hand, in the MDA-MB-435 cell line, cells that express BCRP are fast cycling and are not enriched in breast TICs as compared to BCRP− cells (Patrawala et al. 2005). These data suggest that different mechanisms may play roles to mediate TIC chemoresistance in different models and that there may be a trade-off between slow cycling and drug efflux properties in conferring drug resistance.

Resistance to radiotherapy

Malignant cells are usually rapidly dividing, and their DNA damage repair systems frequently fail to perform dependably (Nie 2012). Therefore, malignant cell growth can be effectively controlled by radiotherapy. More than 50% of breast cancer patients receive radiotherapy during the treatment of their disease (Langlands et al. 2013). Unfortunately, distant metastasis and local recurrence still occur because of treatment resistance (Langlands et al. 2013), especially in patients with basal-like TNBC (Nguyen et al. 2008).

Accumulating evidence suggests that breast TICs are radioresistant (Phillips et al. 2006, Woodward et al. 2007, Prat et al. 2010, Morrison et al. 2011). For example, when they are isolated from p53-null mouse mammary tumors carried by irradiated mice, the CD24+/CD29− subpopulations, which are cell types enriched for TICs, resolve their γH2Ax DNA damage foci more rapidly than non-TIC subpopulations do; this suggests that mouse mammary TICs have a more effective DNA repair system compared to non-TICs from the same tumor (Zhang et al. 2010). Furthermore, in the human breast cancer cell line MCF7, radiation enriches for breast TIC subpopulations (Phillips et al. 2006, Woodward et al. 2007). Similarly, in two patient-derived TNBC xenograft models (MC1 and BCM-2665A), radiation has been shown to enrich for the ALDH+ subpopulation (Atkinson et al. 2010). In addition, clinical data have shown that triple-negative breast tumors, which include the breast TIC-enriched claudin-low subtype, are generally more resistant to radiotherapy as compared to other types of tumors (Prat et al. 2010). Together, these data suggest that breast TICs are more resistant to radiotherapy. However, the resistance of breast TICs to radiation is not without controversy. One study showed a depletion of TICs in one patient-derived xenograft after radiation, as measured by the percentage of CD44+/CD24−/low/Lin− cells, ALDH1 levels, and MSFE, but the opposite was observed in a second independent PDX model (Zielske et al. 2011). This controversy may be the result of the use of inappropriate breast TIC markers, or it may indicate that some breast tumors have a radiosensitive TIC population.

Resistance to endocrine therapy

Endocrine therapy drugs, such as tamoxifen and aromatase inhibitors, have been the cornerstone of treating ER+ breast cancer and have significantly decreased mortality (Jensen & Jordan 2003, Johnston & Dowsett 2003,
Dowsett et al. 2005). However, disease recurrence occurs in up to 25–30% of the patients within 5 years following tamoxifen treatment (Early Breast Cancer Trialists’ Collaborative Group (EBCTCG) 2005, Howell & Wardley 2005). For patients with recurrence, de novo and acquired resistance have become major challenges for successful treatment (Early Breast Cancer Trialists’ Collaborative Group (EBCTCG) 2005). A range of mechanisms have been postulated to account for tamoxifen resistance, from a lack of ERα expression (Clarke et al. 2003) to an increased expression of growth factor tyrosine kinases (Knowlden et al. 2003, Dowsett et al. 2006, Massarweh et al. 2006, Giltnane et al. 2007).

The role of breast TICs in endocrine therapy resistance is controversial. On the one hand, even in ER+ tumors, breast TICs are predicted to be ER−, because CD44+ cells are ER− and the expression of ALDH1 is inversely correlated with ER and PR expression (Clarke et al. 1997, Ginestier et al. 2007, Shiptsin et al. 2007, Harrison et al. 2013), which suggests that breast TICs themselves would not respond to endocrine therapy directly. On the other hand, estrogen has been shown to expand the ER− breast TIC population via paracrine FGF/Tbx3 or EGFR/Notch signaling (Fillmore et al. 2010, Harrison et al. 2013), which suggests that they could be indirectly sensitive to endocrine therapy. However, a recent study provided evidence to support the predicted resistance of ER− cells to endocrine therapy by showing the expansion of the ER−/PR− subpopulation in ER+/PR+ breast cancers in response to antiestrogen treatment (Haughian et al. 2012). This result suggests that ER− breast TICs are likely to be resistant to endocrine therapy.

Resistance to anti-HER2 therapy

About 15–20% of breast cancer patients have breast tumors of the HER2+ subtype, which means that the tumor cells have overexpression typically accompanied by HER2 gene amplification (Slamon et al. 1987, Allred 2010). HER2-targeting drugs, such as trastuzumab, greatly improve the prognosis of patients with HER2+ breast cancer. However, 50% of recurrences are the result of de novo resistance and, of the patients with HER2+ metastatic tumors that do respond to trastuzumab initially, the majority acquire resistance within 1–2 years of treatment (Lan et al. 2005, Chung et al. 2013).

Although anti-HER2 agents have been shown to target breast TICs with, or sometimes without, HER2 amplification (Korkaya et al. 2009, Magnifico et al. 2009, Ithimakin et al. 2013), several lines of evidence support a role for breast TICs in trastuzumab resistance: i) HER2 overexpression expands the breast TIC population in vitro and in vivo (Korkaya et al. 2008, Cicalessi et al. 2009). ii) Signaling pathways that are known to generate trastuzumab resistance also expand the breast TIC population (Korkaya et al. 2012, Chakrabarty et al. 2013, Hanker et al. 2013, Ding et al. 2014). iii) Long-term trastuzumab treatment of resistant cells enriches the breast TIC population as measured by TIC markers (Reim et al. 2009, Korkaya et al. 2012). Taken together, these results suggest that combining breast TIC-targeting agents with HER2-targeting agents will benefit patients that do not respond to anti-HER2 therapies alone.

Plasticity of breast TICs and potential therapeutic implications

There is evidence to suggest that breast TICs and non-TICs are interconvertible, either spontaneously or through induction (Meyer et al. 2009, Gupta et al. 2011, Iliopoulos et al. 2011, Kim et al. 2013). The apparent plasticity of breast TICs has also been hypothesized to be an obstacle in treating breast cancer. Although facilitating the transformation of breast TICs to breast non-TICs can be an effective therapeutic strategy, the opposite transformation would obviously be problematic (Visvader & Lindeman 2012). Indeed, it has been reported in breast cancer cell lines that a subset of cells in certain phenotypic states will eventually return to proportions of phenotypic equilibrium by stochastically transitioning between states following the Markov model, which also predicts that breast TICs and non-TICs are capable of converting to each other (Gupta et al. 2011). In support of this model, it has been shown that in multiple breast cancer cell lines, a single CD44+/CD24− cell gives rise to the CD44+/CD24− progeny in vitro and that xenograft tumors generated by CD44+/CD24− cells are similar to tumors initiated by CD44+/CD24− cells (Meyer et al. 2009). Likewise, another study showed that in transformed MCF10A cells, the CD44+/CD24−/low stem-like subpopulation is able to rapidly convert back to other cell types until it reaches an equilibrium proportion in which 10% of its cells are similar to those of the parental cell line and that the non-TICs can give rise to breast TICs in vivo, which demonstrates that interconversion between these cell states is possible (Iliopoulos et al. 2011). Furthermore, in transformed human mammary epithelial (HMLE) cells, CD44low cells convert spontaneously into CD44+ cells in vitro and in vivo (Chaffer et al. 2011), which demonstrates another case of breast TIC plasticity.
The underlying mechanism of such conversions also emerged recently, and it was found to be associated with the EMT program. For instance, knocking down FOXC2 leads to inhibition of mesenchymal phenotype and reduction in tumor-initiating capacity in breast cancer cell lines (Hollier et al. 2013). Furthermore, it has been shown that the ZEB1 promoter undergoes conformational changes in response to the TGFβ signal to drive breast cancer cell plasticity (Chaffer et al. 2013). Similarly, the conversion of the luminal-like CD44+ /CD24− cells into basal-/mesenchymal-like CD44+/CD24− cells depends on the Activin/Nodal-initiated TGFβ signaling (Meyer et al. 2009). These studies have been performed predominantly in cell line models, and the degree to which plasticity occurs in vivo in PDX models has not been established.

This ‘plastic CSC’ phenomenon may add additional layers of complexity to the treatment of breast cancer. In particular, if TICs can be generated de novo from non-TICs within a tumor, therapies designed to target TICs may ultimately fail. In that case, targeting both TICs and non-TICs is imperative. Targeting the de-differentiation mechanism, which could result from gene mutations, epigenetic modifications, or stochastic events, is also promising (Marjanovic et al. 2013).

**Metastatic potential of breast TICs and therapeutic implications**

The proposed enhanced metastatic potential of breast TICs may be another treatment hurdle. Because TICs have the ability to generate tumors at orthotropic sites, it has been hypothesized that they could also generate tumors at metastasis sites (Brabletz et al. 2005). For example, it has been shown that spontaneous lung metastases and primary breast tumors share similar CD44+ profiles that are enriched for breast TICs (Liu et al. 2010), which suggests that TICs are involved in spontaneous metastasis. Furthermore, ER− metastatic tumors have been found in ER+ patients, which demonstrates the metastasis potential of ER− TICs (Lower et al. 2005, Fehm et al. 2008). The EMT program is thought to enable the invasion of tumor cells into the stroma, and it therefore may initiate the early steps of the metastatic process (Scheel & Weinberg 2012).

Importantly, the EMT program has been coupled with breast TIC formation. For instance, human mammary epithelial cells (MECs) undergoing EMT also show enhanced TIC properties (Mani et al. 2008), and the expression of EMT regulators forces non-TICs into the TIC state (Li et al. 2009, Chaffer et al. 2013, Hollier et al. 2013). Moreover, following chemotherapy, residual breast cancer cells display EMT features (Creighton et al. 2009), and knockdown of TWIST reverses chemotherapy-induced MDR and EMT concurrently (Li et al. 2009), which indicates the formation of drug-resistant breast TICs during EMT. Additionally, MET, a reverse process of EMT, is thought to be essential during the last step of metastasis (Scheel & Weinberg 2012). Contrary to the established pro-EMT signaling, which involves TGFβ and TWIST, TGFβ and Id1 signaling has been reported to induce the MET and stem-like phenotypes in breast cancer cells by targeting TWIST (Stankic et al. 2013). Together, these data link breast TIC formation to both EMT and MET programs, and they suggest the metastatic potential of TICs.

Breast TICs were also thought to possess enhanced metastasis capacity. For example, CD44+ /CD24− human breast cancer stem-like cells not only express EMT markers (Mani et al. 2008), but they also display an increased incidence and burden of metastasis when they are administrated through tail vein injection (Croker et al. 2009). In addition, ALDH expression is associated with the MET state, and ALDH activity is largely a result of the ALDH1A3 isoform that is associated with metastasis (Marcato et al. 2011, Liu et al. 2014). The metastatic potential of TICs provides insight into the mechanism of cancer progression. Targeting TICs may therefore help eradicate primary tumors and prevent metastasis simultaneously. However, because of the complicated involvement of some common programs in both the MET and the EMT processes, considerable caution is required when attempting to pinpoint the different progression stages in each patient.

**Pathways that regulate breast TICs and confer resistance**

TICs may survive conventional therapy and contribute to recurrence and metastasis later, even in a rapidly shrinking tumor. Therefore, a combination of drugs that target TICs and non-TICs and a combination of parameters that measure tumor volume and TIC functions are likely required for future clinical studies. In order to develop TIC-targeting drugs, the signaling pathways that are utilized by breast TICs and therapeutic agents that target those pathways are currently being intensively studied.

**Hedgehog signaling**

Hedgehog (HH) was first identified in a genetic screen for the genes required for *Drosophila* embryonic patterning
(Driever & Nusslein-Volhard 1988). In mammals, HH signaling functions in multiple tissue/cell types in the developing embryo to direct organogenesis, including the ventral–dorsal pattern formation in the neural tube and the anterior–posterior pattern formation in the limb (Ingham & McMahon 2001). Despite the debate about its functional significance in postnatal mammary gland development (Lewis & Visbal 2006), paracrine HH signaling has been shown to stimulate proliferation and to expand the progenitor population in the mouse mammary gland in transgenic mice (Visbal et al. 2011, Garcia-Zaragoza et al. 2012). Furthermore, activation of HH signaling promotes mammosphere formation of normal mammary stem cells, whereas inhibition of HH signaling by cyclopamine exerts the opposite effects, which suggests that HH signaling plays a role in normal mammary stem cells (Liu et al. 2006, Moraes et al. 2007). Importantly, HH signaling is activated in the Lin−/CD44−/CD24−/low human breast TIC subpopulation, and it promotes mammosphere formation in the p53-null mouse mammary tumor model through Bmi-1, a polycomb protein that is overexpressed in the Lin−/CD29+/CD24−/low subpopulation (Liu et al. 2006, Zhang et al. 2008). More recently, it was reported that GLI1, a downstream mediator of HH signaling, stimulates tumor initiation in triple-negative breast tumors (Goel et al. 2013), and GLI1 is required for breast TIC self-renewal in ER− breast cancer cells (Sun et al. 2014). These findings imply that HH signaling components could be targets for treating breast TICs.

WNT signaling

Wnt1 was originally identified as a proto-oncogene because it was retrieved from an oncogenic integration site of MMTV (Nusse & Varmus 1982), and Wnt1 transgenic overexpression generates mammary tumors in mice within 6 months (Tsukamoto et al. 1988). Wnt signaling is required for normal mammary stem cell function and Wnt-responsive cells show enriched stem cell activity in the mammary gland (Andl et al. 2002, van Amerongen et al. 2012). Therefore, Wnt1-induced tumor formation may be a result of the ability of Wnt signaling to transform mammary stem cells (Li et al. 2003, Zeng & Nusse 2010). Wnt-responsive cells are also enriched in TICs in mouse mammary tumors (Zhang et al. 2010), which are located close to distorted blood vessels (Zhu et al. 2013, Vadakkan et al. 2014); this suggests that WNT signaling plays a role in breast TICs. Accordingly, a WNT inhibitor decreases the TIC frequency of an RAS-transformed mesenchymal subpopulation of human MECs (Scheel et al. 2011). Importantly, WNT signaling plays a pivotal role in the radioresistance of breast TICs. For example, TICs isolated from p53-null mouse mammary tumors have enriched Wnt signaling activity and more effective DNA damage repair systems (Zhang et al. 2010). In addition, clinically relevant doses of radiation specifically enrich the SP and the Sca1+ stem-like subpopulations in MECs that have activated Wnt signaling but not in WT MECs (Woodward et al. 2007). Finally, radiation enriches for Sca1+ cells, and these cells show elevated Wnt signaling activity and fewer γH2AX DNA damage foci as compared to Sca1− cells (Chen et al. 2007). Together, these findings suggest that WNT signaling inhibitors have the potential to sensitize resistant breast TICs to radiotherapy. Recent studies by Gunther et al. reported that both the luminal and basal populations were required for efficient tumor formation in the MMTV-driven Wnt1 genetically engineered mouse model and were dependent on luminal Wnt1 expression (Cleary et al. 2014).

WNT/β-catenin signaling is activated by the binding of a WNT ligand to its receptor, Frizzled, and its co-receptor, lipoprotein receptor-related protein 5/6 (LRP5/6), which makes Frizzled and LRP5/6 candidate targets for inhibiting WNT signaling (MacDonald et al. 2009). Salinomycin inhibits WNT/β-catenin signaling by inducing LRP6 degradation, and it has also been identified as a selective inhibitor of breast TICs in a high throughput screen (Gupta et al. 2009, Lu et al. 2011, Lu & Li 2014). A number of natural dietary components, including curcumin, piperine, and sulforaphane, have also been found to inhibit breast TICs and to down-regulate the WNT pathway (Kakarala et al. 2010, Park et al. 2010). Despite this progress, the development of potent WNT inhibitors remains challenging (Anastas & Moon 2013). Interestingly, pSTAT3 binds to the promoter region of β-catenin directly and activates β-catenin transcription in MCF7 and BT474 breast cancer cell lines, and pSTAT3 and β-catenin staining are significantly correlated in primary breast tumors (Armanious et al. 2010). Investigating the crosstalk between STAT3 and WNT signaling may potentially provide novel intervention targets for inhibiting WNT signaling.

NOTCH signaling

In vertebrates, there are four transmembrane Notch receptor proteins: Notch1–4. Notch1 is a proto-oncogene that is capable of inducing mouse mammary tumors when it is abnormally expressed by MMTV insertion (Gallahan & Callahan 1987, 1997). Notch pathway activators promote,
whereas Notch pathway inhibitors suppress, the formation of secondary mammospheres from normal mammary stem cells, which suggests that Notch signaling plays an important role in normal stem/progenitor cell function (Dontu et al. 2004). Moreover, both NOTCH1 and NOTCH4 antibodies decrease the MSFE of breast tumor cells derived from PDX or patient samples, which suggests their function in breast TICs (Farnie et al. 2007, Qiu et al. 2013). Importantly, NOTCH4 signaling activity is eightfold higher in the ESA+/CD44+/CD24low TIC subpopulation, and NOTCH4 inhibition reduces the TIC frequency in human breast cancer cell lines in vivo (Harrison et al. 2010). More recently, it was found that in basal-like breast cancer, NF-κB induces the expression of JAG1, a Notch ligand, in non-TICs, and this stimulates NOTCH signaling in TICs in trans, which leads to breast TIC expansion (Yamamoto et al. 2013). Another study showed that NOTCH signaling is activated in ER− cells, whereas it is inhibited in ER+ cells by estradiol, which makes NOTCH signaling a potential target for overcoming endocrine therapy resistance in ER− cells, the putative breast TIC population (Rizzo et al. 2008).

NOTCH signaling is activated by γ-secretase cleavage of its intracellular domain. Therefore, γ-secretase inhibitors (GSIs) have been used as pan-NOTCH signaling inhibitors (Bray 2006). For example, the GSI MK-003 was shown to successfully eliminate TICs in a mouse model of ErbB2 breast cancer, as measured by LDT assays (Kondratyev et al. 2012). More recent and promising data suggest that the MK-003 reduces breast TICs in patient-derived xenograft tumors and that MK-0752, an MK-003 analog, may reduce breast TICs in patient biopsies in a phase I clinical trial, as measured by MSFE and the expression levels of stem cell markers (Schott et al. 2013). However, it has also been shown that NOTCH4 knockdown is more efficient than GSIs are in targeting TICs; this is likely because NOTCH4 activity is increased and NOTCH1 activity is decreased in breast TICs (Harrison et al. 2010). Interestingly, a recent study showed that in some TNBC lines, the activated NOTCH1-ICD is only increased in the CD44+/CD24low breast TICs, but not in the CD44+/CD24− breast TICs. Therefore, the CD44+/CD24low stem-like population is sensitive to GSIs, but the CD44+/CD24− subpopulation, which is also stem-like, is resistant (Azzam et al. 2013). These data suggest the heterogeneous nature of NOTCH signaling within breast TICs and indicate that, similar to other proposed breast cancer therapies, GSIs may need to be combined with other treatments to maximize their efficiency.

Interleukin 6/STAT3 signaling

STAT3 belongs to a family of latent transcription factors (Chatterjee-Kisshore et al. 2000), and it plays a pivotal role in early embryogenesis by acting to keep ESCs in an undifferentiated state (Matsuda et al. 1999, Raz et al. 1999, Torres & Watt 2008). Recent evidence suggests that it also plays a role in regulating stem cells in solid tumors, including breast tumors. For example, in breast cancer models, treatment with the STAT3 pathway agonist interleukin 6 (IL6) expands the CD44high/CD24low stem-like subpopulation (Iliopoulos et al. 2011), whereas shRNA-mediated STAT3 knockdown decreases the TIC frequency (Zhou et al. 2007). Additionally, in patient breast tumor samples, the majority of CD44high/CD24low cells have been shown to be positive for pSTAT3 staining (Marotta et al. 2011). A more recent report linked high STAT3 activity to high autophagy function, which contributes to chemoresistance in breast cancers (Maycotte et al. 2014). Taken together, these data suggest that STAT3 is associated with breast TIC function.

Recently, our laboratory showed that STAT3 signaling is activated preferentially in TICs of claudin-low-type human breast cancer, which further suggests that STAT3 signaling regulates breast TIC function (Wei et al. 2014). Consistent with these data, a recent study showed that the anti-malarial drug chloroquine inhibits Jak2/STAT3 signaling, decreases the MSFE in a number of TNBC cell lines, and decreases the TIC frequency in MDA231 xenograft tumors (Choi et al. 2014). Importantly, it has also been shown that breast TIC-derived IL6 recruits mesenchymal stem cells, which communicate with breast TICs by secreting additional cytokines and chemokines, thereby creating a breast TIC-promoting microenvironment (Liu et al. 2011). Furthermore, an inflammatory feedback loop has been shown to enhance breast TIC function; in this feedback loop, breast cancer cells under long-term trastuzumab treatment are highly enriched in the CD44+/CD24− population and secrete high levels of IL6, which in turn acts through the IL6 receptor to expand the CD44+/CD24− stem-like population (Korkaya et al. 2012).

STAT3 signaling is typically induced by the binding of IL6-type cytokines to gp130 receptors, and this binding activates JAKs (Yu & Jove 2004). Therefore, STAT3 signaling can be inhibited by JAK inhibitors and small molecules that directly target STAT3. The former is exemplified by ruxolitinib, which is currently being tested in phase II trials to treat breast cancer (Quintas-Cardama & Verstovsek 2013). The latter is exemplified by piperlongumine, which has been shown to block the mammosphere
formation of patient-derived xenograft tumor cells and to inhibit breast tumor growth in vivo (Bharadwaj et al. 2014). Other in vivo studies have used STAT3 inhibitors, including Cmp188, a competitive small-molecule inhibitor, to compete with STAT3 to bind pY peptides (Xu et al. 2009). In addition, chloroquine, an anti-malarial drug, has been reported to inhibit JAK/STAT3 signaling and to reduce the CD44+/CD24−/low subpopulation in triple-negative breast tumors (Choi et al. 2014).

Another method for inhibiting STAT3 signaling includes blocking the IL6 receptor. For example, a combined treatment using an anti-IL6 receptor antibody and trastuzumab reduces the TIC frequency in trastuzumab-resistant HER2+ breast cancer cells (Korkaya et al. 2012). Together, these data suggest that STAT3 inhibitors would be promising therapeutic drug candidates for targeting both breast TICs and their niches.

**IL8/CXCR1/2 signaling**

CXCR1 is a member of the G protein-coupled receptor family, and it is highly expressed in ALDH+ cells in a number of breast cancer cell lines (Charafe-Jauffret et al. 2009). Treatment with the CXCR ligand IL8 increases the ALDEFLUOR+ populations and the MSFE in breast cancer cell lines (Charafe-Jauffret et al. 2009, Singh et al. 2013), whereas treatment with a CXCR1 inhibitor depletes the breast TIC subpopulation (Ginestier et al. 2010). IL8 is also secreted by mesenchymal stem cells in response to signals from breast TICs, and it reinforces breast TIC function (Liu et al. 2011). More recently, it was reported that IL8/CXCR1/2-induced mammosphere formation is mediated partially by EGFR/HER2 signaling (Singh et al. 2013), which provides a rationale for using a combined therapeutic strategy. Indeed, the small molecule CXCR1 inhibitor repertaxin has been shown not only to effectively target breast TICs but also to enhance the efficacy of lapatinib in treating HER+ breast cancers (Singh et al. 2013).

IL8 is the best-studied CXCR1/2 ligand, and antibodies against IL8 are currently being tested in clinical trials for the treatment of inflammatory diseases (Skov et al. 2008). However, the benefits of inhibiting IL8 may be limited, because a broad range of other ligands can also stimulate CXCR1/2 (Bieche et al. 2007). Therefore, blocking CXCR1/2 function provides much more specificity. For instance, repertaxin shows potent inhibition of breast TICs in vitro, and it reduces tumor growth and metastasis in vivo (Ginestier et al. 2010). It also has an additive effect when used in combination with lapatinib to treat HER+ breast cancers (Singh et al. 2013), and it has undergone clinical testing in combination with chemotherapy to treat patients with HER2− metastatic breast cancer (Schott et al. 2012).

**TGFβ signaling**

TGFβ family members are multifunctional peptides that regulate cell growth and differentiation (Heldin et al. 1997). TGFβ signaling has also been shown to maintain the undifferentiated state of human ESCs (James et al. 2005). The role of TGFβ signaling in mammary carcinogenesis is controversial, because it is known to impair tumorigenesis, but it also triggers the EMT/MET program to facilitate metastasis (Siegel et al. 2003, Muraoka-Cook et al. 2005, Tang et al. 2007, Stankic et al. 2013). Importantly, TGFβ and its receptor are specifically expressed in CD44+ cancer cells, and their expression results in a more mesenchymal appearance of those cells (Shipitsin et al. 2007). Together, these data link TGFβ signaling to a mesenchymal/stem-like state of breast cancer cells. Surprisingly, a recent finding suggested that TGFβ/Id1 signaling also induces MET and stem-like phenotypes in breast cancer cells, and this only occurs in cells that have already undergone an EMT. Hence, TGFβ is predicted to facilitate metastasis during both the EMT and MET steps in a sequential manner (Stankic et al. 2013). More recently, an oscillating TGFβ3–JUND signaling circuit was uncovered in basal-like breast cancer cells, which suggests that this is an additional underlying mechanism for intratumoral heterogeneity (Wang et al. 2014b). All of these findings highlight a context-dependent role of TGFβ in breast TIC plasticity and metastasis.

Several examples illustrate the importance of TGFβ signaling in chemotherapy-induced breast TIC enrichment and metastasis. In the 4T1 mouse mammary tumor model, the chemotherapy drug doxorubicin induces EMT and promotes a stem-like phenotype by activating TGFβ signaling, which is effectively blocked by a TGFβ type 1 receptor kinase inhibitor (TβRI-KI). Combining doxorubicin with TβRI-KI significantly reduces tumor growth and lung metastasis in vivo (Bandypadhyay et al. 2010). Similarly, the chemotherapy drug paclitaxel enriched for breast TICs in TNBCs, as indicated by MSFE assay and TIC markers (Bhola et al. 2013), and this effect can be prevented by treating with LY2157299, a small molecule TβRI-KI (Bhola et al. 2013). However, considering the multiple functions of TGFβ in the plasticity and metastasis of breast TICs (Stankic et al. 2013, Wang et al. 2014b), additional caution is required when targeting this signaling pathway.
Integrin signaling

Integrins are environmental sensors and, importantly, are differentially expressed between breast TICs and non-TICs. For example, the β3 subunit integrin CD61 has been used to enrich a breast TIC subpopulation in MMTV-Wnt1 mouse mammary tumors, because the CD29<sup>lo</sup>/CD24<sup>low</sup>/CD61<sup>+</sup> subset shows increased TIC frequency than the CD29<sup>lo</sup>/CD24<sup>+</sup>/CD61<sup>−</sup> subset does (Vaillant et al. 2008). Furthermore, the α6 integrin subunit CD49f is a biomarker for TICs in ER<sup>−</sup> and TNBC xenografts (Meyer et al. 2010, Lee et al. 2014). Recently, an autocrine loop between VEGF and integrin signaling has been uncovered in breast TICs. In this loop, VEGF signaling activates α6β1 (CD49fCD29) integrin, which enhances the expression of Gli and leads to the transcription of the VEGF receptor neuropilin 2 (Goel et al. 2013). Another study demonstrated that VEGF signaling transcriptionally regulates an RNA-splicing factor that regulates the splicing of α6β1 (CD49fBCD29) integrin. This splice form of α6β1 (CD49fCD29) integrin defines a mesenchymal-like subpopulation within the CD44<sup>high</sup>/CD24<sup>low</sup> cells, and it is critical for breast TIC function (Goel et al. 2014). Also recently, the integrin α(v)β3 (α(v)CD61) and the KRAS/NFKB signaling pathway were shown to drive resistance to EGFR inhibition in breast TICs (Seguin et al. 2014), which suggests that integrin proteins have a function in breast TICs.

In addition, focal adhesion kinase (FAK) is a major mediator of integrin signaling. Targeted deletion of Fak in mouse mammary epithelium decreases the expression of ALDH<sup>+</sup> stem-like populations in primary MMTV-PyMT mammary tumors, and it decreases the tumorigenicity of ALDH<sup>+</sup> cells in vivo (Luo et al. 2009). Functional studies show that integrin signaling actively maintains the mammary cancer stem/progenitor population (Luo et al. 2009). Further studies have suggested that FAK plays a distinct role in mammary stem cells and progenitors and that it plays correspondingly different roles in claudin-low- and luminal-like-type breast cancer cells, which suggests a potential relationship between normal stem cells/progenitors and different subtypes of breast cancers (Luo et al. 2013). Together, these recent discoveries of detailed mechanisms that involve integrin signaling in breast TICs provide novel intervention opportunities.

Treatment with anti-β1 integrin antibodies enhances the response of human xenograft breast tumors to radiotherapies (Park et al. 2008) and normalizes structures of breast cancer cell lines in 3D cultures (Park et al. 2006). In addition, the recent finding that integrin signaling in breast TICs drives resistance to EGFR inhibitors suggests potential novel strategies for sensitizing breast tumors to receptor tyrosine kinase inhibition (Seguin et al. 2014). Cilengitide, an integrin antagonist currently being tested in clinical trials (Goodman & Picard 2012), has been shown to reduce bone metastasis in a mouse mammary tumor model (Bauerle et al. 2011). A more recent study also reported that the increased stiffness of the extracellular matrix in tumors can activate integrin signaling to drive tumor progression, which suggests another new intervention method (Mouw et al. 2014).

MicroRNAs

MicroRNAs (miRNAs) are short, noncoding RNAs that bind to complementary sequences in mRNAs, which results in the suppression of translation and/or degradation of targeted mRNAs (Bartel 2009). Importantly, more than 30 miRNAs have been reported as being differentially expressed between breast TICs and non-TICs (Shimono et al. 2009), which suggests that miRNAs may function to regulate breast TICs. Indeed, let-7 family members are down-regulated in mammospheres formed from primary breast tumor cells as compared to bulk tumor cells, and forced expression of let-7a reduces MSFE (Yu et al. 2007). miR-200 family members are also down-regulated in CD44<sup>+</sup>/CD24<sup>−</sup> cells, and forced expression of miR-200c reduces tumor-initiating capacity (Shimono et al. 2009). Interestingly, miR-93 is highly expressed in non-TICs in claudin-low-type breast cancer, but it is not differentially expressed between TICs and non-TICs in luminal-type breast cancer. Forced expression of miR-93 decreases the TIC frequency in claudin-low-type tumors, whereas it increases the TIC frequency in luminal-type tumors, which suggests that the same miRNA can play distinct roles in regulating TICs in different types of breast cancer (Liu et al. 2012). Moreover, Lin28, a negative regulator of let-7, is a direct target of the WNT-β-catenin pathway. Loss of function of Lin28 reverses WNT-mediated let-7 inhibition and breast TIC expansion (Cai et al. 2013). These data suggest that miRNAs can be mediators of WNT-regulated breast TIC function, and as such, they are promising targets for inhibiting breast TIC expansion. Taken together, these data suggest that miRNAs could be useful agents to target for the treatment of breast cancer.

Inhibition of miRNAs can be achieved by i.v. injection of antagonirs, which are modified antisense oligos that are complementary to the miRNA sequence (Krutzfeldt et al. 2005). In one example, anti-let-7 oligos were introduced...
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<td>Decrease</td>
<td>Decrease</td>
<td></td>
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<tr>
<td></td>
<td>MCF7</td>
<td>shNotch4</td>
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<td>0.125 µM</td>
<td>Decrease</td>
<td>Decrease</td>
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<td>IL6/STAT3</td>
<td>MMTV-ErbB2 tumor</td>
<td>MRK-003</td>
<td>JAG1</td>
<td>0.01 µM</td>
<td>Decrease</td>
<td>Decrease</td>
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<td>Decrease</td>
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<td></td>
<td>HCC1973</td>
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<td></td>
<td>Phase I patients</td>
<td>Stage 3 295</td>
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<td>10 µM</td>
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<td>Decrease</td>
<td>Bharadwaj et al. (2014)</td>
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<td></td>
<td>PDX, SUM149 xenograft</td>
<td>shSTAT3</td>
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<td>10 µM</td>
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<td>Decrease</td>
<td>Wei et al. (2014)</td>
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<tr>
<td>IL8/CXCR1/2</td>
<td>MDA231/SUM159 xenograft cells (STAT3–GFP&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Stattic</td>
<td></td>
<td>5 µM</td>
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<td></td>
<td>HCC1954, SUM159, MDA453</td>
<td>IL8</td>
<td></td>
<td>50–100 ng/ml</td>
<td>Increase</td>
<td>Decrease</td>
<td>Charafe-Jauffret et al. (2009)</td>
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<td></td>
<td>SUM159, HCC1954</td>
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<td>Patient samples</td>
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<td>Increase</td>
<td>Decrease</td>
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into breast cancer cells, which resulted in the enhanced self-renewal of non-TICs (Yu et al. 2007). Alternatively, synthesized artificial miRNAs that mimic the effect of a miRNA can be introduced. For instance, when let-7 mimics were administered to mouse models of lung cancer, they led to reduced tumor growth (Esquela-Kerscher et al. 2008).

However, targeting breast TICs by manipulating miRNAs remains challenging. One problem is that each miRNA potentially regulates hundreds of genes simultaneously, which leads to poor specificity. Also, cancer cells tend to produce mRNAs with shorter 3′-UTRs, which often contain the miRNA target sites, and that makes them resistant to miRNA mimics (Mayr & Bartel 2009).

Panels of inhibitors/activators have been developed for each of the signaling pathways mentioned earlier in the present report. To evaluate their proposed effects on TICs, MSFE, and LDT assays should be applied to measure their efficiency. We provided a summary of the activatory and inhibitory agents that target those signaling pathways in Table 3, emphasizing their effectiveness on TIC function.

### Summary

The hypothesized existence of resistant breast TICs provides a potential mechanism for explaining treatment failure, recurrence, and metastasis in the treatment of breast cancer using current therapeutic methods. Therefore, inhibiting the pathways that are essential for breast TIC function is a promising strategy for overcoming drug resistance. Furthermore, combining conventional therapies with breast TIC-targeting drugs should improve long-term patient outcome by eliminating TICs, which potentiate future recurrence and metastasis. Moreover, effective therapeutic strategies should address concerns regarding the dynamic properties of breast TICs, such as their plasticity and their enhanced metastatic potential, which could become problematic during breast cancer treatment. Finally, although simply targeting one major signaling pathway is unlikely to eliminate breast TICs completely, sequential or simultaneous administration of inhibitors that target multiple signaling pathways may render long-lasting and synergistic effects on TICs. Alternatively, given our success in eliminating non-TICs, inducing the differentiation of TICs to other cell types by manipulating essential signaling pathways, and then employing conventional therapies, could be a safe and effective strategy. Nonetheless, genome instability in stem-like cells predicts that TICs are evolving entities that quickly adapt to alternative survival mechanisms. We
will have to continue to build on our knowledge about breast TICs by revealing the novel mechanisms and alternative signaling pathways that are essential for their maintenance and function.

Declaration of interest
M T Lewis is a scientific founder and limited partner in StemMed Ltd and is a manager of StemMed holdings LLC.

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