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Cancer stem cells and early stage basal-like breast cancer

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Abstract

Ductal carcinoma *in situ* (DCIS) is a category of early stage, non-invasive breast tumor defined by the intraductal proliferation of malignant breast epithelial cells. DCIS is a heterogeneous disease composed of multiple molecular subtypes including luminal, HER2 and basal-like types, which are characterized by immunohistochemical analyses and gene expression profiling. Following surgical and radiation therapies, patients with luminal-type, estrogen receptor-positive DCIS breast tumors can benefit from adjuvant endocrine-based treatment. However, there are no available targeted therapies for patients with basal-like DCIS (BL-DCIS) tumors due to their frequent lack of endocrine receptors and HER2 amplification, rendering them potentially susceptible to recurrence. Moreover, multiple lines of evidence suggest that DCIS is a non-obligate precursor of invasive breast carcinoma. This raises the possibility that targeting precursor BL-DCIS is a promising strategy to prevent BL-DCIS patients from the development of invasive basal-like breast cancer. An accumulating body of evidence demonstrates the existence of cancer stem-like cells (CSCs) in BL-DCIS, which potentially determine the features of BL-DCIS and their ability to progress into invasive cancer. This review encompasses the current knowledge in regard to the characteristics of BL-DCIS, identification of CSCs, and their biological properties in BL-DCIS. We summarize recently discovered relevant molecular signaling alterations that promote the generation of CSCs in BL-DCIS and the progression of BL-DCIS to invasive breast cancer, as well as the influence of the tissue microenvironment on CSCs and the invasive transition. Finally, we discuss the translational implications of these findings for the prognosis and prevention of BL-DCIS relapse and progression.

Keywords

Ductal carcinoma *in situ*; Invasive ductal carcinoma; Basal-like ductal carcinoma *in situ*; Basal-like invasive ductal carcinoma; Cancer stem cells

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INTRODUCTION

Breast cancer has been recognized as a complex, heterogeneous disease, encompassing multiple cell populations with different risk factors, histological features, clinical behaviors and responses to therapy[1-3]. While breast cancer diagnosis was initially based on tumor size, histological classification systems were later developed to categorize breast tumors into subgroups. Primary breast carcinomas are first classified as either *in situ* or invasive tumors. Ductal carcinoma *in situ* (DCIS) of the breast is an early stage, non-invasive breast tumor commonly diagnosed by mammography screening. DCIS accounts for 15%-20% of all newly diagnosed breast cancer cases, and is characterized by the intraductal proliferation of malignant epithelial cells without invasion through the basement membrane into the surrounding tissue[4]. In contrast, invasive breast carcinoma is able to invade through the basement membrane into the surrounding stroma. Invasive breast carcinomas have been extensively studied and found to be composed of numerous heterogeneous histological subtypes[5]. Similar to invasive breast carcinoma, divergent histological types of DCIS lesions have been recognized, and various classification systems have been developed to characterize DCIS tumors[6]. Approximately 15%-30% of DCIS patients relapse within 10 years after surgical lumpectomy[7], and it is of urgent clinical need to have an effective classification system to identify DCIS with a high-risk of tumor recurrence. Owing to subjective interpretation of lesion morphology by pathologists, inconsistency in DCIS classification cannot be avoided[8]. Histological classification of heterogeneous DCIS lesions is not sufficient to identify molecularly heterogeneous DCIS subgroups, and additional classification approaches are necessary for the pathological characterization of DCIS and identification of more effective therapeutic options.

Gene expression profiling has emerged as a useful system for breast cancer classification[9-11], and has been used to define five intrinsic molecular subtypes of invasive breast carcinoma: Luminal A, luminal B, HER2-enriched, normal- and basal-like[9,10]. Following this discovery, additional subgroups of breast cancer were identified, including the interferon-enriched[12], molecular apocrine[13] and claudin-low subgroups[14]. Given that these subtypes possess different molecular alterations, they display distinct clinical outcomes and therapeutic responses. The basal-like subtype is highly aggressive and therefore of particular clinical relevance. Basal-like breast cancers are more likely to occur in younger, African American women, and are associated with breast cancer susceptibility (*BRCA*) gene mutations. They are characterized by high tumor grade, proliferation rate, frequency of recurrence, and the presence of *p53* mutations. Patients with basal-like breast cancers frequently have poor prognosis, and are difficult to treat due to the lack of effective targeted therapies[10,11,15]. Breast tumors categorized as basal-like display gene expression signatures similar to normal basal/myoepithelial breast cells (myofibroblast-like breast epithelial cells located between breast ductal epithelial cells and the basement membrane), including high-molecular weight basal cytokeratins (CK5/6, CK14 and CK17)[16].

The majority of diagnosed basal-like breast cancer cases are triple-negative breast cancers (TNBC), which lack expression of hormone receptors [estrogen receptor (ER) and progesterone receptor (PR)] and overexpression/amplification of HER2[9,10,15]. Although there is significant overlap between basal-like breast cancers and TNBC, they are not

identical. Approximately 70%-80% of basal-like breast cancers have been identified as triple-negative, basal-like breast cancer (TN-BLBC)[15,17,18]. The remaining non-triple-negative basal-like breast cancers share similar gene expression profiles with TN-BLBC, but might have gained additional genetic and/or epigenetic aberrations due to increased genomic instability[17,18]. Using gene expression profiling analysis, Lehmann *et al*[19] identified six distinct molecular subtypes of TNBC: Two basal-like (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like and luminal androgen receptor (LAR). Among these TNBC molecular subtypes, BL1, BL2, IM and M are predominantly basal-like[20]. This sub-classification of TNBC is clinically relevant due to the differential clinical outcome and chemotherapeutic response of each TNBC subtype[20,21]. Two lines of evidence indicate that distinct molecular subtypes of TNBC manifest differential responses to immunoediting, the process by which tumor cells escape the anti-tumor effect of immunosurveillance[22,23]. Similar to Lehmann's sub-classification of TNBC, Burstein *et al*[22] utilized RNA and DNA profiling analysis to define TNBC subtypes as: (1) LAR; (2) mesenchymal (MES); (3) basal-like immunosuppressed (BLIS); and (4) basal-like immune-activated (BLIA). BLIS tumors have the worst prognosis and BLIA tumors manifest the best[22], in part due to the ability of the immune system to target them. Similarly, Jézéquel *et al*[23] sub-classified TNBC into three different subtypes *via* gene-expression profiles, including the C1 subtype (LAR, 22%), the C2 subtype (basal-like with a low immune response and high M2-like macrophages, 45%) and the C3 subtype (basal-enriched with a high immune response and low M2-like macrophages, 33%). They also found that basal-enriched C3 with a high immune response had a better prognosis than basal-like C2 with a low immune response[23]. While the molecular mechanisms leading to immune tolerance are not fully understood, two lines of study indicate that inactivation of tumor suppressor p53 and activation of CUL4A E3 ubiquitin ligase are involved in failure of tumor immunosurveillance[24,25].

TN/BLBC are more sensitive to preoperative or neoadjuvant chemotherapy than luminal breast cancers. Current therapeutic options for TN/BLBC include cytotoxic (*e.g.*, combined treatment with anthracyclines or taxanes) and targeted (*e.g.,* PARP1 and EGFR inhibition) therapies[20,21,26]. Although cytotoxic therapeutics achieves good tumor regression rates in the neo-adjuvant setting, patients experience frequent recurrence in five years after treatment[26,27]. Targeted therapies have also encountered discrepancies in trial results and issues with resistance[26,27]. TN/BL drug resistance potentially involves several mechanisms including intrinsic therapy resistance by a minor cell population in tumors, therapy-induced senescence and polyploidy in tumor cells, acquired therapeutic resistance *via* upregulation of drug efflux transporters, and acquired resistance *via* genetic reversion[28]. It is well accepted that tumor-initiating cells [generally called cancer stem cells (CSCs), discussed in the following paragraph] have high intrinsic drug-resistance, and may lead to relapse[28]. In regard to cell-cycle-related mechanisms, Puig *et al*[29] reported that cisplatin treatment induced senescent giant polyploid cells *via* DNA endoreduplication. These giant, multi-nucleated cells were able to generate small-sized, diploid cells that started to proliferate and were increasingly cisplatin-resistant[29]. These findings suggest that the multistep drug-resistant progression in which cells undergo DNA endoreduplication, polyploidization, depolyploidization and then generation of clonogenic escape cells, can account for tumor

relapse after initial efficient chemotherapy[29]. Another common mechanism of drug resistance in cancer is the upregulation of ATP-binding cassette (ABC) transporter family proteins, which increases the efflux of chemotherapeutic drugs[28]. Besides these mechanisms, genetic alterations to restore the function of DNA repair proteins (*e.g.*, BRCA1, BRCA2, FANCA, *etc.*) have been identified as a novel drug-resistant mechanism in DNA-repair-deficient cancers, which are initially sensitive to DNA-damaging agents (*e.g.,* cisplatin) and to PARP inhibitors[28]. In addition to these general resistance mechanisms, dysregulation of signal pathway regulators in basal-like breast cancers have recently been identified to be responsible for resistance to neoadjuvant chemotherapy and PARP inhibitor treatment. For example, basal-like breast cancers have low expression of dual specificity protein phosphatase 4 (DUSP4), which negatively regulates the Ras-ERK pathway, due to hypermethylation of the DUSP4 promoter. This results in activation of the Ras-ERK pathway and resistance to neoadjuvant chemotherapy[30]. Furthermore, overexpression of p38 mitogen-activated protein kinase in basal-like breast cancers enabled cancer cells to be resistant to the treatment of PARP inhibitors[31]. These results show the promise of increased molecular and functional characterization of TNBC subtypes. While the development of therapeutics to effectively treat TNBC still faces tremendous challenges, molecular targeting of TNBC based on molecular subtypes is a promising therapeutic strategy.

A conceptual model has been proposed that describes the developmental process of breast cancer as the progression of atypical hyperplasia into carcinoma *in situ* and finally to invasive carcinoma[32]. To better understand the relationship between DCIS and invasive breast carcinoma in breast cancer progression and to molecularly classify DCIS lesions, immunohistochemical and gene expression profiling analyses were used to define DCIS subtypes. The molecular subtypes of invasive breast cancer are also present at the DCIS stage, although their frequencies are varied between these two distinct breast cancer stages[33-35]. Furthermore, genetic studies of DCIS and invasive breast carcinoma have demonstrated that they have remarkable similarity in their genetic profiles when matched by histological grade and hormone receptor status[3,36-38]. These findings strongly support the theory that DCIS is a non-obligate precursor of invasive breast cancer. The distinct tumor subtypes may be generated from different cells of origin, by distinct tumor progression pathways, or a combination of both events. The ability of DCIS to progress to invasive disease is a complex biological phenomenon and depends on multiple factors, including genetic/epigenetic aberrations, genomic instability and the stromal microenvironment[3,32].

As poorly differentiated invasive ductal carcinomas (IDC), basal-like breast tumors presumably have a DCIS precursor with similar cytologic and immunophenotypic features. Several studies reported that the use of both gene expression profiling and immunohistochemical analysis of basal-specific protein markers identified DCIS tumors with molecular features of basal-like invasive breast cancer[33,39-43]. These basal-like DCIS (BL-DCIS) tumors are presumed to be precursors for basal-like IDC (BL-IDC)[39,44]. Identification of BL-DCIS sheds light on the possibility of preventing progression to malignant basal-like breast cancer through the therapeutic targeting of precursor DCIS lesions. This review summarizes the recent investigation of BL-DCIS characteristics and the potential precursor relationship between BL-DCIS and invasive basal-like breast carcinoma.

CSCs have been identified in many types of cancer including breast cancer, and have significantly changed the strategy of cancer therapy. CSCs are a small tumor cell subpopulation that can be identified by several methods including fluorescence-activated cell sorting (FACS) analysis of stem/progenitor-cell-specific surface protein markers, aldehyde dehydrogenase (ALDH) activity assays, and FACS analysis of the “side population” indicated by Hoechst dye exclusion. CSCs have the unique ability to self-renew and to differentiate into heterogeneous tumor cell lineages *in vitro* and *in vivo*[45-48]. CSCs possess higher tumorigenic ability than non-CSCs, which can be measured by *in vivo* xenograft tumor formation assays involving the injection of enriched CSC fractions to immunodeficient mice[45-48]. Similar to normal stem cells, CSCs manifest stem-cell-specific gene expression signatures and can undergo symmetric as well as asymmetric cell division[45-48]. CSCs also exhibit high levels of drug resistance. Under the stress of chemotherapy they can become quiescent and resistant to drugs that target proliferating cells[49,50]. In addition, CSCs generally express high levels of multi-drug resistant ABC transporters and pump out anti-cancer drugs at high rates[51]. The stem-like characteristics of CSCs are due to dysregulation of stemness signaling pathways, such as the Notch, hedgehog, Wnt, TGF-β and pluripotent transcription factor (*e.g.,* SOX2, OCT4, KLF4, *etc.*) pathways[52,53]. Moreover, CSCs possess similar characteristics to cells that have undergone epithelial-to-mesenchymal transition (EMT), allowing CSCs to survive in circulation and contribute to the metastasis of invasive cancers[54,55]. Due to these traits, CSCs are believed to be necessary for tumor heterogeneity, relapse, metastasis, and drug resistance. Breast CSCs were first identified in primary breast carcinomas using the markers $CD44^+$/$CD24^-$[56]. This minor tumor cell subset can self-renew to form tumorspheres in *in vitro* suspension culture conditions, and exhibit stem-cell gene expression patterns[56]. Isolated $CD44^+/CD24^-$ cells have a higher capacity to initiate *in vivo* xenograft mammary tumors compared with other cell subsets and can differentiate into heterogeneous breast tumor cell lineages[56]. Furthermore, breast CSCs display a drug-resistant phenotype, and are able to better tolerate anti-cancer drug treatment than non-CSCs[57,58]. Enrichment of CSCs in breast cancer correlates with tumor aggressiveness, likely due to the characteristics described above. Among the molecularly-classified breast cancer subtypes, basal-like breast carcinomas tend to possess the highest proportion of CSCs compared with other subtypes, consistent with their heterogeneity and aggressiveness[59,60]. CSCs in basal-like breast cancer have emerged as a key target for cancer therapy.

These discoveries have prompted cancer researchers to investigate the existence of CSCs and their characteristics in BL-DCIS[61,62]. Studies employing *in vitro* cell line and *in vivo* xenograft tumor models have significantly propelled the understanding of CSC characteristics and their role in BL-DCIS. They also give new insights into the aberrant molecular mechanisms involved in regulating CSC formation in BL-DCIS and the BL-DCIS-to-IDC transition. This article will review recent advances in these topics and their translational implications to the prognosis and prevention of BL-DCIS progression to invasive basal-like breast cancer.

EXISTENCE AND FEATURES OF BASAL-LIKE-DCIS

Due to the lack of effective targeted therapies, invasive basal-like breast cancers have poor prognosis. This has prompted cancer researchers to investigate the existence of precursor DCIS lesions that can potentially develop into BL-IDC. If precursor DCIS lesions with the potential to develop into BL-IDC are identified, patients with these lesions can be treated earlier with more aggressive therapies to prevent tumor progression and recurrence. Precursor DCIS lesions are presumed to have cytologic and immunophenotypic features similar to BL-IDC. By characterizing protein markers such as ER, PR, HER2, basal cytokeratins (*e.g.,* CK5/6, CK14, and CK17), EGFR, c-kit and p63, about 6%-8% of DCIS cases were identified to be TN/BLBC[39-41,43]. In addition to immunohistochemical surrogates, Hannemann *et al*[33] performed microarray-based gene expression profiling to analyze and classify 40 *in situ* and 40 invasive breast cancer cases. Their two-dimensional hierarchical clustering analysis of microarray data showed that the luminal, HER2 and basal-like subtypes originally described in invasive breast cancer could also be identified in DCIS. A population-based cohort has shown that patients with BL-DCIS have a higher risk for local recurrence and development into invasive cancer compared with other molecular subtypes[63], demonstrating the need for further molecular characterization. The BL-DCIS subtype is associated with unfavorable prognostic variables such as high-grade nuclei, mutant p53 overexpression and elevated Ki-67 index[42]. In addition, through RNA deep sequencing analysis, Abba *et al*[64] subdivided high-grade DCIS into two subtypes, DCIS-C1 and DCIS-C2. The more aggressive DCIS-C1 (highly proliferative, basal-like, or ERBB2$^+$) had a molecular signature characteristic of activated regulatory T (Treg) cells (CD4$^+$/CD25$^+$/FOXP3$^+$) and CTLA4$^+$/CD86$^+$ complexes, indicative of a tumor-associated immunosuppressive phenotype[64]. This is the first evidence identifying mechanisms of immune evasion in BL-DCIS. Recently BL-DCIS tumors have also been associated with cell cycle-related biomarkers[65,66]. Over 80% of BL-DCIS cases were p16-positive, whereas over 90% of DCIS cases with the luminal A phenotype were p16-negative[66]. In addition to p16 expression, co-expression signatures of p16$^+$/Ki67$^+$/COX2$^+$ and p16$^+$/Ki67$^+$/COX2$^-$ were also found to be associated with the basal phenotype in DCIS and IDC[66]. These cell-cycle related profiles could be exploited to guide more aggressive treatment strategies in patients with high-grade DCIS. According to studies by Tamimi *et al*[42], the frequency (7.7%) of BL-DCIS in diagnosed DCIS cases is slightly lower than that (10.7%) of BL-IDC in diagnosed invasive breast cancer cases. One plausible explanation for the slightly higher frequency of basal-like expression in high-grade invasive *vs in situ* tumors is either that BL-DCIS lesions rapidly progress, leading to the lower identifiable frequency, or that the basal-like phenotype is acquired during invasive progression. Studies investigating the precursor potential of comedo-DCIS tumors (comedo-DCIS), a type of high-risk *in situ* breast lesions, identified a novel p63/CK5/Her2/neu-expressing cell subpopulation with ER-/PgR-/EGFR-[67]. Given that p63 alone and p63/Her2/neu co-expression are both associated with microinvasion and the recurrence of clinical comedo-DCIS, the p63/Her2/neu-expressing precursor intermediate is considered a cellular basis for the emergence of p63$^+$/Her2/neu$^-$ or p63$^+$/Her2/neu$^+$ basal-like breast cancer, and thus may serve as a biomarker for identifying the BL-DCIS subgroup[67].

CSCS AND BASAL-LIKE-DCIS

Evidence of CSCs existing in DCIS

It has been proposed that CSCs are responsible for generating tumor heterogeneity and the malignant progression of cancer. To validate this hypothesis within breast cancer and investigate the mechanisms of the DCIS to IDC transition, researchers are seeking to identify the existence of CSCs in DCIS and characterize their cell properties. To study the heterogeneous tumorgenicity of cancer cells, Damonte *et al*[68] generated mammary intraepithelial neoplasia (MIN) outgrowth lines. Derived from premalignant atypical lesions from PyV-mT transgenic mice, the MIN outgrowth lines are able to grow orthotopically in cleared mammary fat pads, and form a mammary tumor structure similar to human DCIS.

The 6 MIN lines generated demonstrated a varied ability to progress to invasive carcinoma with pulmonary metastatic potential *via* serial transplantation[68], establishing the paradigm that pre-CSCs in DCIS are capable of self-renewal, multilineage differentiation, and serve as the origin of invasive cancer. Notably, their studies indicate that sequential genetic hits for malignant transformation are not required for this DCIS model to progress to invasive and metastatic mammary carcinoma, and the programmed potential for latency and metastasis might be predetermined in these pre-CSCs[68]. Moreover, Espina *et al*[69] studied *ex vivo* organoid culture of fresh human DCIS lesions without enzymatic digestion or sorting, and found that DCIS contains malignant precursor cells that were able to form spheroids and a duct-like 3D structure in *ex vivo* organoid culture and to exhibit tumorigenicity in NOD/SCID mice[69].

Evidence for the presence of CSCs in BL-DCIS

The two lines of evidence mentioned above raised the possible existence of tumorigenic CSCs in BL-DCIS, which may determine the phenotypes of BL-DCIS and the capability of BL-DCIS to progress into invasive cancer. To confirm this, our research group characterized the BL-DCIS cell model MCF10DCIS.COM, which is derived from the non-cancerous breast epithelial cell line MCF10A. This BL-DCIS-mimic cell model has a unique bipotent progenitor ability, and is able to generate both myoepithelial and luminal-type cells *in vivo*, giving rise to BL-DCIS with high similarities to human DCIS lesions[70-76]. In addition to the formation of DCIS-like tumor structures *in vivo*, these tumor lesions are able to spontaneously progress to invasive breast cancer[74,76]. In our studies of MCF10DCIS.COM, we identified a CSC population with enriched $ALDH1^+$ and the molecular signature $CD44^+/CD49f^+/CD24^-$[62]. Compared with the non-stem-like cell subset, these stem-like cells possessed enhanced migration, invasion and self-renewal capacity, and accelerated xenograft tumor growth in nude mice[62]. Pandey *et al*[61] have also identified CSCs in the MCF10DCIS.COM cell line using similar cell surface markers ($CD44^+/ESA^+/CD24^-$). In line with our result, CSCs isolated using this profile showed significantly higher DCIS tumor-initiating ability compared with non-stem-like cells[61]. The existence of CSCs in BL-DCIS raises the possibility that this CSC population serves as a malignant precursor necessary for the progression of BL-DCIS to BL-IDC.

DEREGULATED FACTORS INVOLVED IN THE GENERATION OF CSCS AND THE BL-DCIS-TO-BL-IDC TRANSITION

A challenging question in the breast cancer research field is how precursor DCIS lesions progress to invasive breast carcinomas. Attempts to address this critical question are hampered by the complexity of heterogeneous DCIS lesions. To overcome this barrier, the aforementioned MCF10DCIS.COM cell line has been extensively exploited as a unique model to study DCIS and explore molecular mechanisms involved in regulating the progression of DCIS to IDC. As MCF10DCIS.COM belongs to the BL-DCIS subtype, we review recent findings of deregulated factors implicated in the generation of basal CSCs in this cell model, and in enhancing malignancies as well as invasive progression of this BL-DCIS model *in vivo*. Further characterization of dysregulated signaling pathways involved in CSCs would advance insights into how BL-DCIS tumors progress to invasive cancer and propel the development of effective therapeutics to prevent this malignant progression.

The role of miR-140

MicroRNAs (miRNAs) are short non-coding RNA molecules with a length of approximately 22 nucleotides that bind to the 3′-untranslated region of messenger RNAs and regulate mRNA stability and/or translation. miRNAs have been extensively investigated in cancer and other diseases, and regulate a variety of physiological and pathological processes at the posttranscriptional level. Although numerous miRNAs have been found to be involved in regulating CSCs in breast cancer[77], the miRNAs participating in basal CSC regulation and the tumorigenic development of BL-DCIS remain largely unknown. Through miRNA profiling of paired DCIS tumors, we identified downregulation of miR-140 as a hallmark of BL-DCIS lesions[78]. Our studies have shown that miR-140 is a tumor-suppressive miRNA which targets the stem-cell related factor SOX9 for degradation in in normal breast epithelial cells[78]. The degree of miR-140 downregulation positively correlates with the increased expression of SOX9 and the grade of DCIS lesions, implicating the critical role of the miR-140/SOX9 axis in the progression of DCIS[78]. Our studies also revealed that miR-140 was downregulated in cancer stem-like $CD44^+/CD24^-$ cells isolated from MCF10DCIS.COM cells compared with normal breast stem cells isolated from MCF10A cells[78]. Moreover, restoration of miR-140 expression in MCF10DCIS.COM cells suppressed CSC self-renewal, invasion and *in vivo* tumorigenicity[78]. This suggests that the miR-140/SOX9 regulatory circuit is pivotal for the self-renewal and invasive capacity of basal CSC and their tumor formation *in vivo*, and is a potential therapeutic target.

The role of the nuclear receptor coactivator amplified in breast cancer 1

Ory *et al*[79] found that expression of nuclear receptor coactivator amplified in breast cancer 1 (AIB1) was aberrantly upregulated in DCIS lesions compared with normal breast.

AIB1 activates NOTCH, HER2 and HER3 signaling pathways in MCF10DCIS.COM cells, and is required for the malignant phenotype of MCF10DCIS.COM in 3D culture and *in vivo* tumor formation and progression[79]. Critically, AIB1 inhibition led to a significant reduction in the $CD44^+/CD24^-$ CSC population and also resulted in decreased myoepithelial progenitor cells in DCIS lesions *in vitro* and *in vivo*[79]. These data indicate that activation

of AIB1 is an aberrant mechanism that initiates and maintains DCIS *in vivo* by facilitating the development as well as maintenance of basal CSCs. It is likely that aberrantly activated AIB1 assists other deregulated factors to promote the transition of BL-DCIS to BL-IDC.

The role of the p63-membrane-type 1-matrix metalloproteinase axis

A critical step in the progression from DCIS to the invasive lesion is the crossing of the basement membrane and invasion into the stroma. This is achieved through degradation of extracellular matrix (ECM) proteins in the basement membrane by membrane-anchored matrix metalloproteinases (MMPs), including membrane-type 1 (MT1)-MMP[80]. MT1-MMP has been shown to be involved in invasive tumor growth and metastasis in several experimental cancer models[81-84]. Lodillinsky *et al*[85] analyzed expression of MT1-MMP in a large cohort of DCIS, IDC and microinvasive breast tumors, and found that MT1-MMP was significantly upregulated in the DCIS to IDC transition, and correlated with higher grade and hormone receptor-negative tumors. Functional analysis showed that silencing of MT1-MMP in MCF10DCIS.COM cells impaired the ability of this DCIS tumor model to progress into infiltrating lesions *in vivo*[85]. Additionally, Lodillinsky *et al*[85] identified p63 as an upstream positive regulator that increases MT1-MMP expression in DCIS, and is required for activating the basement membrane-invasive program of DCIS. Their findings suggest that aberrant activation of the p63/MT1-MMP axis in DCIS may contribute to the progression of DCIS to high-grade basal-like breast cancers. Although their studies did not address the role of the p63/MT1-MMP axis in MCF10DCIS.COM CSCs, p63 is a well-known basal-associated molecular marker, and has been recently found to be elevated in CSCs of HER2-type breast cancer and essential for their self-renewal as well as tumorigenicity[86]. Therefore, their results imply that aberrant activation of the p63/MT1-MMP axis in basal CSCs is a potential mechanism to trigger the progression of BL-DCIS to BL-IDC.

The role of Singleminded-2s

Recent work has demonstrated that the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) transcription factor Singleminded-2s (SIM2s) is critical for normal mammary gland development and promoting tumor cell differentiation[87]. SIM2s is inhibited by C/EBPβ and NOTCH, important promoters of EMT and cell differentiation. Loss of SIM2s enhances EMT in the mouse mammary gland, normal breast and breast cancer cell lines. Moreover, SIM2s is frequently downregulated in human breast cancer. When SIM2s expression was restored in human breast cancer cell lines, their proliferation and invasion were suppressed. These results suggest that SIM2s is a tumor suppressor gene that is crucial for maintaining epithelial integrity through inhibiting the EMT program and promoting cell differentiation. To address the role of SIM2s in the transition of DCIS-to-IDC, Scribner *et al*[87] analyzed SIM2s expression in MCF10DCIS.COM and found that it is downregulated in this DCIS cell model when compared with non-cancerous MCF10A cells. Moreover, their functional studies showed that reestablishment of SIM2s in MCF10DCIS.COM cells significantly impaired their growth and invasion both *in vitro* and *in vivo* by promoting tumor cell differentiation. This is characterized by increased expression of luminal markers, including β-casein, E-cadherin, keratin 18, and decreased expression of genes associated with stem cell maintenance and a basal/EMT phenotype, including smoothened, p63, Snail-2, keratin

14 and vimentin[87]. In contrast, abrogation of SIM2s in MCF10DCIS.COM-derived xenograft tumors led to a more invasive phenotype and increased lung metastasis, correlating with the elevated expression of Hedgehog signaling and MMP[87]. From our and other studies indicating that basal CSCs are the origin of the tumorigenic and invasive characteristics of MCF10DCIS.COM cells[61,62,79], it is likely that decreased expression of SIM2s promotes the development of basal CSCs in BL-DCIS and further reduction in its expression activates invasive features of CSCs to facilitate the invasive progression of BL-DCIS into invasive breast carcinoma.

The role of lipogenesis

Cancer cells have altered metabolisms in comparison to normal cells[88], and upregulation of lipogenic genes and increased lipogenesis are hallmarks of late-stage breast cancer[89]. Inhibition of key lipogenic enzymes results in suppression of tumorigenicity both *in vitro* and *in vivo* by blocking proliferation and inducing apoptosis[90-93]. Although the role of increased lipogenesis in late-stage breast cancer has been extensively studied, its role in early-stage breast cancer DCIS still remains elusive. Moreover, whether lipogenesis is engaged in regulating CSCs of DCIS is an interesting yet unexplored question. To address the role of lipogenesis in DCIS CSCs, Pandey *et al*[61] used the cell surface marker profile ($CD44^+/ESA^+/CD24^-$) to isolate CSCs from the MCF10DCIS.COM cell line for expression analysis of lipogenic genes. Their studies showed that expression levels of all lipogenic genes tested in the CSC population were significantly higher than the normal stem-like counterpart population isolated from non-cancerous MCF10A cells. To further investigate the role of sterol regulatory element-binding protein-1 (SREBP1), the master transcriptional activator of lipogenic genes, in CSCs, SREBP1 was ectopically overexpressed in MCF10A stem-like cells. Overexpression of SREBP1 caused enhanced lipogenesis, cell growth and mammosphere formation[61]. When upregulated in MCF10AT, a MCF10A-derived, premalignant cell line, SREBP1 promoted DCIS generation *in vivo* by increasing CSC survival[61]. These findings indicate that activation of lipogenesis is a pre-requisite for basal CSC generation and DCIS formation, and is important for endowing increased cell survival capacity.

THE IMPACTS OF THE TISSUE MICROENVIRONMENT ON CSCS OF BL-DCIS

DCIS lesions are heterogeneous tumors encapsulated by the myoepithelium and basement membrane. When they progress to IDC, tumor cells cross the myoepithelial layer and basement membrane and invade into the stroma, comprised of stromal fibroblasts/preadipocytes, mature adipocytes, immune and endothelial cells. Therefore, these various tissue cells and the ECM that composes the tumor microenvironment can regulate CSC self-renewal and differentiation, DCIS formation, progression into invasive lesions, and metastasis[94-98]. Understanding the impact of the tumor microenvironment on basal CSCs and BL-DCIS could potentially enable the design of more effective diagnosis and intervention strategies to improve the survival of cancer patients. There are two lines of recent studies indicating the critical impacts of the tissue microenvironment on the

tumorigenesis of BL-DCIS and their transition to BL-IDC, exosomal signaling and ECM dependent signaling.

Exosomal signaling from the tumor microenvironment

Exosomal secretion is a newly identified mechanism of paracrine signaling through which cells secret exosomes, microvesicles with a diameter usually less than 100 nm, which can contain cargo proteins, nucleic acids and nutrients[99]. Secreted exosomes can transduce their carried contents into surrounding cells *via* the cell internalization mechanism mediated by the heparan sulfate proteoglycan receptors[99]. The known roles of exosomes in tumorigenesis are to restructure the tumor tissue microenvironment, modulate tumor immune responses and directly regulate tumor cell behaviors *via* their delivery of proteins and genetic materials. miRNAs have been found to be one kind of nucleic acids carried by secreted exosomes[99,100]. Given that miRNAs are regulatory factors that can modulate protein expression, exosomal trafficking of miRNAs has been recognized to be a microenvironmental signal that can affect signaling networks at the post-transcriptional level[100]. From BL-DCIS studies, we found that the miRNA content in exosomes secreted from CSCs of DCIS was altered compared to exosomes from normal stem-like breast cells[62]. Notably, CSC-secreted exosomes carried less miR-140, an aforementioned tumor-suppressive miRNA, than those secreted from normal stem-like cells, suggesting that the tumorigenic process alters exosomal contents[62].

In addition to the role of exosomal trafficking in signaling among DCIS tumor cells, we recently found that exosomes secreted from preadipocytes, the precursors of mature adipocytes, could impact the stemness and tumorigenic properties of CSCs in BL-DCIS[101]. Preadipocyte-derived exosomes enhanced *in vitro* cell migration as well as self-renewal of BL-DCIS cells and facilitated the xenograft tumor formation of transplanted BL-DCIS cells *in vivo*[101]. The enhanced effect of preadipocyte-secreted exosomes on the tumorigenicity of BL-DCIS might be attributable to a number of growth-promoting cytokines identified within these exosomes[101]. Taken together, these findings demonstrate that exosomal signaling plays an important role in the tumor microenvironment.

ECM-dependent regulatory signaling

High tumor heterogeneity correlates with poor prognosis due to its association with malignancies, recurrence, metastasis and anti-cancer drug resistance[102]. Intratumor heterogeneity could result from an intrinsic stochasticity in gene expression and from genetic and/or heritable epigenetic differences among tumor cells[103]. By studying the effect of ECM on an immortalized basal-like breast epithelial cell line, Wang *et al*[104] identified the ECM-dependent TGFBR3 (transforming growth factor β receptor 3)-JUND (jun D proto-oncogene)-KRT5 (keratin 5) regulatory circuit that generates heterogeneous gene expression among ECM-attached breast cells. This circuit is composed of two anticorrelated gene expression programs that negatively regulate each other. TGFBR3 signaling downregulates JUND mRNA levels, whereas JUND represses both TGFBR3 and JUND mRNA levels[104]. Perturbing this regulatory circuit in breast epithelial cells could lead to the formation of aberrant tissue lesions similar to high-grade DCIS[104]. Their studies also indicate that the TGFBR3-JUND circuit is the molecular mechanism responsible for the

heterogeneous expression of KRT5 in some basal-like premalignant lesions[104]. These findings suggest that heterogeneous KRT5 expression patterns present in high-grade basal-like DCIS lesions are likely due to loss of tissue-level regulation of gene oscillatory networks rather than genetic selection. Disrupting the dependence of this regulatory circuit on ECM results in detachment of breast epithelial cells from the ECM, in turn leading to cell death[104]. However, some cells survive through activation of a juxtacrine tenascin C (TNC) deposition mechanism. TNC is a critical survival factor for detached cells that would otherwise be subjected to keratinization-induced or anoikis-dependent cell death, and participates in stabilizing the heterogeneous JUND-KRT5 expression[102,104]. These results are in line with the previous finding that metastasizing breast cancer cells express TNC to elicit and/or maintain their metastasis-initiating characteristics[105]. Particularly, it has been shown that TNC is able to increase the expression of stem-cell signaling proteins, suggesting its role in modulating the CSC population[105]. Their findings demonstrate that this ECM-dependent regulatory circuit program can maintain normal tissue architecture and function in addition to preventing cell outgrowth and migration when it is properly regulated. However, when dysregulated (*e.g.*, aberrant ECM signaling), this system enables cells to evade keratinization and anoikis, and allows them to metastasize.

THE IMPLICATIONS OF CSCS IN PROGNOSIS AND PREVENTION OF EARLY STAGE BASAL-LIKE BREAST CANCER

The identification of CSCs in BL-DCIS opens a window for cancer researchers to explore how BL-DCIS initiate and progress into invasive basal-like breast cancer. In addition to promoting our understanding of the role of basal CSCs in BL-DCIS, these research advances have tremendous translational implications for future prognostic and therapeutic applications. The dysregulated molecular factors which result in basal CSC generation could potentially be exploited as prognostic biomarkers for BL-DCIS. This would help identify and grade the probability of diagnosed DCIS developing into BL-IDC, and determine whether DCIS patients should be treated more aggressively. If this prognostic system can be established, it will substantially benefit patients with DCIS and lower their chances of basal-like invasive breast cancer recurrence. The therapeutic agents that can target these deregulated factors could be potentially exploited for targeted therapy of DCIS. This chemopreventive strategy would save breast cancer patients' lives, especially since there are currently no effective therapies to cure basal-like invasive breast cancer. Promising therapeutic agents have already been identified that are effective in targeting CSCs in BL-DCIS. The dietary compound sulforaphane (SFN) can restore miR-140 expression and downregulate the expression of miR-140 targets SOX9 and ALDH1, inhibiting the self-renewal of basal CSCs and DCIS formation *in vivo*[62]. Besides SFN, our studies of the chemopreventive agent Shikonin (SK), a bioactive compound found in the herbal plant shikon, showed that exosomes secreted from SK-treated preadipocytes lost the ability to promote BL-DCIS tumorigenicity both *in vitro* and *in vivo*. This is a novel chemopreventive mechanism for BL-DCIS, targeting the tumor microenvironment in place of the tumor itself[101]. Furthermore, a study from Watabe's research group shows that resveratrol, a therapeutic agent capable of blocking the lipogenic gene expression in basal CSCs, is able to significantly suppress DCIS formation in animals[61]. These exciting findings provide a

strong rationale to propel the development of chemopreventive therapeutics for DCIS patients after surgical and radiological treatment.

CONCLUSION

Although the research efforts to combat invasive basal-like breast cancer have provided tremendous insights into this breast cancer subtype, we still have not identified effective therapeutic agents and strategies to cure this disease. Therefore, identifying and targeting the precursor of aggressive breast cancer is a promising direction to prevent the occurrence of this disease. BL-DCIS is an early stage breast cancer with a high risk of recurrence, and targeting it may prevent cancer recurrence and progression to invasive disease. As summarized and discussed in this review, numerous signaling pathways and factors have been identified as dysregulated in basal CSCs of BL-DCIS. Moreover, several chemopreventive agents have been tested to target these deregulated mechanisms. These studies suggest targeting CSCs in BL-DCIS as a potential strategy to inhibit the tumorigenicity of BL-DCIS and prevent the progression of BL-DCIS into BL-IDC. However it is critical to test the proof-of-principle of these chemopreventive strategies in clinical trials. Developing reliable BL-DCIS biomarkers will allow clinicians to design effective targeted therapies that can prevent the recurrence and progression of early stage basal-like breast cancers.

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Core tip

Basal-like ductal carcinoma *in situ* (BL-DCIS) often lacks endocrine receptors and has a high rate of recurrence due to no available targeted therapies. BL-DCIS is a precursor of invasive basal-like breast carcinoma, a malignant cancer prone to metastasis and drug-resistance. Therefore, targeting BL-DCIS to prevent transition into invasive cancer is of significant interest. The recent identification and characterization of cancer stem-like cells in BL-DCIS advance the understanding of BL-DCIS and their potential role in driving the progression of BL-DCIS to invasive basal-like breast cancer. These findings provide critical implications for the development of therapies that prevent the progression of BL-DCIS.