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ER α -Negative and Triple Negative Breast Cancer: Molecular Features and Potential Therapeutic Approaches

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Abstract

Triple negative breast cancer (TNBC) is a type of aggressive breast cancer lacking the expression of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor-2 (HER-2). TNBC patients account for approximately 15% of total breast cancer patients and are more prevalent among young African, African-American and Latino women patients. The currently available ER-targeted and Her-2-based therapies are not effective for treating TNBC. Recent studies have revealed a number of novel features of TNBC. In the present work, we comprehensively addressed these features and discussed potential therapeutic approaches based on these features for TNBC, with particular focus on: 1) the pathological features of TNBC/basal-like breast cancer; 2) E₂/ER β – mediated signaling pathways; 3) G-protein coupling receptor-30/epithelial growth factor receptor (GPCR-30/EGFR) signaling pathway; 4) interactions of ER β with breast cancer 1/2 (BRCA1/2); 5) chemokine CXCL8 and related chemokines; 6) altered microRNA signatures and suppression of ER α expression/ER α -signaling by micro-RNAs; 7) altered expression of several pro-oncogenic and tumor suppressor proteins; and 8) genotoxic effects caused by oxidative estrogen metabolites. Gaining better insights into these molecular pathways in TNBC may lead to identification of novel biomarkers and targets for development of diagnostic and therapeutic approaches for prevention and treatment of TNBC.

Keywords

Breast cancer; CXC chemokine; CXCL8; ER α ; ER β ; estrogen carcinogenesis; GRCP-30/EGFR; mircoRNAs; therapeutic approaches for TN-breast cancer; triple negative breast cancer

1. Pathological features of Triple Negative and Basal-like Breast Cancer

Breast cancer is a heterogeneous disease that encompasses several distinct entities with remarkably different biological characteristics and clinical behaviors. These subtypes of breast cancer are generally diagnosed based upon the presence or absence of three receptors: estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor-2 (HER-2) [1,2]. A large fraction (more than 60%) of total breast cancer patients are ER-, PR- and HER-2 positive. However, there are approximately 15% of all types of breast cancer in

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women who do not express ER α , PR, and HER-2 and, are, thus, defined as triple-negative breast cancer [3].

Estrogens, notably 17 β -estradiol (E₂), are essential for the normal growth and differentiation of human breast epithelial cells. It is generally believed that estrogens regulate cellular responses through binding to two cognate receptors, ER α and ER β , which are ligand-regulated transcription factors with a broad range of physiological functions. In the nucleus, estrogens modulate the expression of estrogen-responsive genes through the action of both ERs at the transcriptional level [4]. Estrogens are also converted to oxidative estrogen metabolites via a number of cytochrome P450s in breast cells. However, prolonged exposure to excess estrogens is a key etiological factor for the development of breast cancer. The carcinogenic effects of E₂ are caused by alterations in ER α - and ER β -mediated nuclear genomic pathways, non-genomic pathways mediated by plasma membrane-associated ERs and by the genotoxic effects resulted from oxidative damage by estrogen metabolites [for review see [5,6]]. There are two forms of progesterone receptor, namely PR-A and PR-B. Both PR-A and PR-B are ligand-activated nuclear transcription factors that mediate progesterone action. Their presence in breast tumors is used to predict functional ERs and, therefore, also to predict the likelihood of response to endocrine therapies and disease prognosis. It has been shown that breast cancer patients with PR-A-rich tumors have poorer disease-free survival rates [7] and that PR-B is involved in regulation of insulin-like growth factor-mediated stimulation of cell migration in breast cancer cells [8]. Progesterone and PRs are essential for estrogen promotion of breast cancer. Over-expression and/or gene amplification of HER-2 are important pathological hallmarks in ER-positive breast cancer patients. It has been known that HER-2 over-expression caused abnormal cellular activities, including un-masking transforming growth factor- β -induced cell mobility [9] and increasing metastatic outgrowth of breast cancer cells in the brain [10]. The current approaches for treating ER-, PR- and HER-2 positive breast tumors include: i) blockage of the interactions between E₂ and ERs with the anti-estrogens, e.g. tamoxifen; ii) inhibition of aromatase-mediated estrogen synthesis with specific inhibitors; and iii) a combination of both i) and ii). In addition, HER-2-based therapies (e.g. trastuzumab therapy) have been applied for treating breast cancer patients with over-expression and/or gene amplification of HER-2.

The term "triple-negative"(TN) breast cancer is used to collectively describe several subgroups of breast cancer patients, including basal-like breast cancer (BLBC) [11] and rare tumors such as metaplastic tumors and adenoid-cystic tumors [12] of the breast whose biology is somewhat different from that of the high-grade invasive ductal TNBC. Thus, TNBC is not a cohesive biologic entity. TNBC patients are different from the ER-, PR- and HER-2 positive breast cancer patients in many ways. Several immunohistochemical and pathological features of breast cancer subgroups are summarized and compared in Table 1, based on references [13, 14]. TNBC are characterized by a number of clinical and histological features [15–19]. The TNBC-subgroups share the following features: a) TNBC is more prevalent among young African, African-American [17,18] and Latino women [17,19], accounting for approximately 25% of all breast cancer patients originating from these ethnic groups. The prevalence of TNBC is also higher among young obese women [19–21]; b) Because of the lack of ER, PR and HER-2, TNBC patients are insensitive to most currently available hormonal or ER-targeted and HER-2-based therapies. They are managed only with standard chemotherapy treatment, which leave them associated with a high rate of local and systemic relapse; c) TNBC patients share several features with breast cancer susceptibility gene outcome-associated breast cancer [22–25]. More than 80% of BRCA-1 mutation carriers are triple negative and approximately 20% of breast cancer patients with mutations in BRCA-1 and BRCA-2 have deficiency in DNA repair, because normal BRCA1 and BRCA2 have an important role in DNA repair. TNBC patients who have problems with DNA repair make the mutation carriers more sensitive to DNA damaging agents; d) TNBC are poorly differentiated, highly malignant, more aggressive,

and with a poor outcome [3,15]. Women with TNBC are twice as likely as other women to develop distant metastases. Because of these, TNBC patients have shorter survival; and e) Altered expression of a number of proteins, oncogenes, tumor suppressor proteins and abnormal signaling pathways has been detected in TNBC (see sections 2–8).

Basal-like breast cancer (BLBC) is characterized by an expression signature similar to that of the basal myoepithelial cells of the breast that express basal-markers including cytokeratins (CKs, e.g. CK5/6, CK17, CK14), epidermal growth factor receptor (EGFR) and myoepithelial makers [26–28]. Clinically, a TN-phenotype definition is commonly used to identify BLBC. However, it should be noted that while both TNBC and BLBC share a number of molecular and morphological features, they are not totally identical. BLBC have transcriptome characteristics similar to those of tumors arising from BRCA1 germline mutation carriers. They are associated with aggressive behavior, poor prognosis, worse overall and disease-free survival compared with those in the luminal A subtype [28]. It has been shown [26] that BLBC defined by five biomarkers (ER-, PR-, HER-2-, EGFR+, and cytokeratin 5/6+) has more superior prognostic value than that of TN-phenotype. Gene expression profiling has allowed classification of breast cancers into five subtypes based upon distinctive gene expression signatures [29]. A better understanding of the molecular and histopathological features of TNBC and BLBC is of paramount importance, in particular for unraveling the heterogeneous nature of these tumor subgroups and for the identification of prognostic biomarkers, ideal systemic therapy regimens and novel therapeutic targets for these aggressive tumors [11].

Currently, the ER -targeted and Her-2-based therapies are not effective against TNBC and BLBC, though several potential approaches for TNBC treatment have been suggested [3,16, 30]. In spite of above described knowledge, the pathogenesis of TNBC is still largely unknown, presenting a major challenge in development of therapies effective for treatment of this type of breast cancer. Gaining better insights into the pathology, new molecular signatures and their prospective validation is essential for designing optimal treatment for TNBC.

2. Existence of E₂/ER β -Mediated Signaling Pathways in TN-Breast Cancer Cells

Two forms of estrogen receptors, namely ER α and ER β , are currently known. ER α is regarded as one of the most important classifiers in breast cancer. Its expression levels govern estrogen-dependent growth, response to endocrine therapy, and prognosis in ER α -positive breast cancer. Both ER α and ER β are co-expressed in large fraction of normal and breast cancer tissues as well as a number of breast cancer cell lines.

However, some breast cells express only ER α whereas other breast cells express only ER β [31]. There are also breast cells that express neither ER α nor ER β [31]. Several studies [32–38] have indicated that ER β acts as an antagonist against ER α -mediated cell proliferation and the expression of a number of gene networks in breast cancer cells that express both ER α and ER β . However, there are data [39–42] indicating that ER β functions differently when it is co-expressed with ER α from when it is expressed alone. Indeed, Zhang et al. [43] revealed that proliferative effects of estrogen in human non-small cell lung cancer cells that expressed ER β but not ER α were mediated primarily, if not exclusively, by the non-genomic, cytoplasmic action of ER β .

The ER status in breast cancer patients has been traditionally defined by the presence or absence of ER α . The ER-negative status in TNBC patients is actually negative to ER α but not necessarily negative to ER β [42]. While TNBC cells do not express ER α , they are estrogen responsive. Estrogens trigger carcinogenic effects in these cells. For example, it has been demonstrated [44] that increasing levels of circulating estrogens were sufficient to promote the formation and progression of ER α -negative cancers whereas pharmacological inhibition of

estrogen synthesis following pregnancy prevented ER α -negative tumor formation. Moreover, the effects of estrogen were shown to act via a systemic increase in host angiogenesis, in part through increasing mobilization and recruitment of bone marrow stromal derived cells into sites of angiogenesis and to a growing tumor mass. These observations suggest that estrogen may promote the growth of ER α -negative breast cancers by acting on cells distinct from the cancer cells to stimulate angiogenesis [44]. The E₂-mediated carcinogenic effects in these cells are mediated via the pathways rather than ER α -mediated pathways.

Several studies [45–49] have reported expression of ER β in a substantial fraction of ER α -negative and TNBC patients. In the United States, approximately 10,000 women are annually diagnosed with ER α -negative/ER β -positive genotype [45]. Gruvberger-Saal et al. [45] examined ER α and ER β expression in 353 primary breast tumors at stage II from patients who were treated with TAM for 2 years and observed that ER β was significantly associated with increased distant disease-free survival (DFS) and that ER β was an independent marker within the ER α -negative tumors. Furthermore, ER β -mediated gene expression profile was markedly different from the ER α -mediated gene signature.

Breast cancer patients who carry mutations in BRCA1 gene are more frequently ER α -negative. However, TAM has a protective effect in preventing contralateral tumors in BRCA1 mutation carriers. This suggests that other mediators are involved in regulation of estrogen's effects in these patients. Litwiniuk et al. [46] examined ER α , ER β and PR in 48 women with mutations in BRCA1 gene and in a control group of 120 breast cancer samples using antibodies for ER α , PR and ER β . Their data revealed that only 14.5% of BRCA1-related cancers expressed ER α compared with 57.5% in the control group. On the contrary, the 42% of BRCA1-related tumors expressed ER β protein compared with 55% in the control group. More importantly, most BRCA1-associated cancer patients were triple-negative but almost half of this group (44.4%) expressed ER β . These observations indicate a more prevalent expression of ER β than ER α in BRCA1-associated tumors.

Novelli et al. [48] examined ER β expression in 936 breast cancers, and found that: i) ER β was evenly distributed across luminal A (LA) and luminal B (LB), HER-2 and TN subtypes; ii) ER β was present in the quadrant containing more aggressive phenotypes such as HER-2, TN and ER α /PR/bcl2-negative tumors; and iii) ER β was a significant discriminating factor for disease-free survival both in the node-negative, LA subgroup, where it is predictive of response to hormone therapy, and in the node-positive LB group where, in association with PR negativity, it conveys a higher risk of relapse. These data indicate that, in contrast to node-negative, the positive ER β expression in node-positive breast cancer patients appears to be a biomarker related to a more aggressive breast cancer.

The wild-type ER β (designated ER β 1) co-exists with four ER β variants (designated ER β 2 to ER β 5) in normal breast and breast cancer cells. The existence of these ER β variants complicates elucidation of their physiological role and involvement in estrogen carcinogenesis. Previous results about the prognostic significance of ER β in breast cancer are conflicting, and this may be due to the differential contributions of ER β 1 and ER β variants. However, several studies [47,50–52] have suggested potential prognostic significance of ER β and ER β variants in breast cancer. Poola et al. [50] investigated the expression of ER β 1 and ER β 5 in ER α -negative breast cancer tissues. They reported that ER α -negative breast tissues expressed significant levels of ER β 1 and ER β 5, and that their expression levels were not different from those in ER α positive tumors. Moreover, there were significant differences between African American and Caucasian groups in that the African American patients expressed higher levels of ER β 1 and ER β 5 but not ER α . Skliris et al. [41] revealed that approximately 60% of ER α -negative tumors were positive for ER β 1 and ER β 2, respectively. Total ER β and ER β 1 were significantly correlated with the cell proliferation marker, Ki67, and with CK5/6 (a marker of BLBC).

ER β 2 was strongly associated with p-c-Jun and NF- κ Bp65. The expression of individual ER β forms was associated with certain phenotypes, suggesting different roles in subsets of ER α -negative cancers. Furthermore, it has been demonstrated [40] that ER β specifically regulated E₂-induced expression of psoriasin/S100A7, an oncogene, both in vitro and in vivo. These observations suggest that ER β may have the potential to become a therapeutic target in the specific sub-cohort of ER α -negative breast cancers and that psoriasin/S100A7 could be useful to guide therapies targeting ER β in certain phenotypic subsets of breast cancers.

Mandusic et al. [46] measured the expression of ER β 1 and ER β 5 mRNAs in the 60 breast cancer samples and correlated their expression with ER α and PR protein levels with clinical/histopathological parameters. They found an inverse correlation between ER β 5 expression and the levels of ER α and PR proteins in postmenopausal patients. The levels of ER β 1 and ER β 5 mRNAs were lower in the larger tumors (>20 mm, T2, and T3) than in smaller ones (< or =20 mm, T1). The decreased ER β 5 mRNA expression in larger tumors was found to arise from ER-positive breast carcinomas. In addition, the portion of tumors with concomitant high expression of both ER β 1 and ER β 5 transcripts matched up the known percentage of tumors resistant to endocrine therapy in patients with different ER/PR status. The higher expression of ER β 5 mRNA was associated with the indicators of low aggressiveness of tumor, suggesting that the uncontrolled local tumor growth may occur as the expression of ER β 5 mRNA decreased in estrogen-dependent breast cancer.

Shaaban et al. [52] stained the breast tissue microarrays with antibodies specific for ER β 1, ER β 2, and ER β 5 in a large cohort of breast carcinomas with long-term followup and scored them as percentage of positive tumor cells using the Allred system. Nuclear and cytoplasmic staining was evaluated and correlated with OS and DFS. They observed that nuclear ER β 2 and ER β 5, but not ER β 1, significantly correlated with OS, and ER β 2 with DFS. ER β 2 also predicted response to endocrine therapy and correlated positively with ER α , PR, androgen receptor, and BRCA1; and also correlated inversely with metastasis and vascular invasion. Tumors that co-express ER β 2 and ER β had better OS and DFS. However, cytoplasmic ER β 2 expression, alone or combined with nuclear staining, predicted significantly worse OS. Notably, patients with only cytoplasmic ER β 2 expression had significantly worse outcome. This study elucidates the prognostic role of ER β 1, ER β 2, and ER β 5 in a large breast cancer series, i.e. ER β 2 is a powerful prognostic indicator in breast cancer, but nuclear and cytoplasmic expression differentially affect outcome. Measuring these parameters in clinical breast cancer could provide a more comprehensive picture of patient outcome, complementing ER α

Honma et al. [51] observed that positive ER β 1 staining was significantly associated with better survival. By contrast, ER β 2 status did not influence survival. In multivariate analysis, ER β 1 status emerged as an independent predictor of recurrence and mortality. ER β 1 status was significantly associated with survival in postmenopausal, but not pre-menopausal, women. Importantly, ER β 1 positivity was significantly associated with better survival in patients with ER α -/PR-negative or TN-tumors, which are widely believed to be hormone unresponsive, have poor prognosis, and require chemotherapy. Thus, examination of ER β 1 in addition to ER α and PR is clinically important in patients with breast cancer treated with TAM.

The studies described above indicate that ER β 1 and other ER β variants are expressed in a fraction of ER α -negative and TNBC cells and suggest that ER β 1 and ER β variants may play some roles in estrogen signaling and the pathogenesis of TNBC. However, the data regarding relationship between the status of ER β and ER β isoforms in ER α -negative and TNBC cells with clinical and histopathological parameters are somewhat inconsistent among different studies. The precise roles of ER β and ER β variants in estrogen-signaling pathways and pathogenesis of TNBC are still not clear. Further studies using ER β -selective agonists, such as diarylpropionitrile [53], 200070 [54] and MF-101[55], and ER β -specific shRNAs [56] are

needed to determine the precise role of each ER β isoforms, their subcellular localization, ER β -mediated signaling pathways and whether alterations in these pathways contribute to the pathogenesis of TNBC. Such studies are valuable for identification of specific downstream markers of ER β -mediated activity in TNBC [42].

3. GPCR-30/EGFR Signaling Pathways and Therapeutic strategies targeting GPCR-30/EGFR Signaling Pathways in TN-Breast Cancer Cells

3.1. GPCR-30/EGFR Signaling Pathways in TN-Breast Cancer Cells

In addition to ER α and ER β , a number of cellular receptor proteins are also involved in mediating the biological effects triggered by estrogens. The G-protein-coupled receptor-30 (GPCR-30) is such an important one that is involved in regulation of several E₂-mediated pathways via epidermal growth factor receptor (EGFR). The EGFR comprises a family of four structurally similar tyrosine kinases with a complex link to downstream signaling molecules that ultimately regulate key cell processes. EGFR family of receptor tyrosine kinases functions as a common signaling conduit for membrane receptors such as G-protein coupled receptors and integrins. High levels of both EGFR and cytokeratin CK5/6 (a BLBC marker) have been detected in the majority of TNBC patients, who have usually high-grade tumors of ductal histology with a high proliferation rate [57,58].

It has been shown that E₂ rapidly activated EGFR to mitogen activate protein kinase (MAPK) signaling pathway. This action required GPCR30, and occurred via G β / γ -subunit-dependent transactivation of EGFR through the release of EGF from the cell surface in ER α -negative human breast cancer cells [59]. Filardo et al. [60] Investigated the mechanism by which Erk-1/-2 activity was rapidly restored to basal levels after E₂ stimulation. They observed that attenuation of Erk-1/-2 activity by E₂ occurred via GPCR30-dependent stimulation of adenylyl cyclase and cAMP-dependent signaling that resulted in Raf-1 inactivation. E₂ repressed EGF-induced activation of the Raf-to-Erk pathway in human breast carcinoma cells that expressed GPCR30, including MCF-7 and SKBR3 cells which expressed both or neither, ERs, respectively. MDA-MB-231 cells, which expressed ER β but not ER α and low levels of GPCR30 protein, were unable to stimulate adenylyl cyclase or promote estrogen-mediated blockade of EGF-induced activation of Erk-1/-2. Pretreatment of MDA-MB-231 cells with cholera toxin, which ADP-ribosylated and activated G α subunit proteins, resulted in GPCR30-independent adenylyl cyclase activity and suppression of EGF-induced Erk-1/-2 activity. Transfection of GPCR30 into MDA-MB-231 cells restored their ability to stimulate adenylyl cyclase and attenuated EGF-induced activation of Erk-1/-2 by E₂. Moreover, GPCR30-dependent, cAMP-mediated attenuation of EGF-induced Erk-1/-2 activity was achieved by ER antagonists e.g. tamoxifen or ICI 182, 780 but not by 17 α -E₂ or progesterone. These results delineate a novel mechanism, requiring GPCR30 and E₂, acting to regulate Erk-1/-2 activity via an inhibitory signal mediated by cAMP. E₂ via GPCR30 stimulated adenylyl cyclase and cAMP-mediated attenuation of the EGFR-to-MAPK signaling axis [60]. GPCR30-mediated trans-activation of EGFRs by E₂ provides cross talk between E₂ and growth factors, and explains the EGF-like effects of E₂. Activation of growth factor-dependent signaling by E₂ has implications for breast cancer biology [58,60].

GPCR30 has all the binding and signaling characteristics of a membrane ER (mER) [61]. A high-affinity, limited capacity, displaceable, single binding site specific for estrogens were detected in plasma membranes of SKBR3 breast cancer cells that expressed GPCR30 but lack nuclear ERs. Progesterone-induced increases and small interfering RNA (siRNA)-induced decreases in GPCR30 expression in SKBR3 cells were accompanied by parallel changes in specific E₂ binding. Plasma membranes of human embryonic kidney 293 cells transfected with GPCR30, but not those of un-transfected cells, and human placental tissues that expressed

GPCR30 also displayed high-affinity, specific E₂ binding typical of mERs. E₂ treatment of transfected cell membranes caused activation of a stimulatory G protein that was directly coupled to the receptor, indicating GPCR30 is a GPCR that also increases adenylyl cyclase activity. The finding that the antiestrogens tamoxifen and ICI 182,780 have high binding affinities to this receptor and mimic the actions of E₂ has important implications for both the development and treatment of estrogen-dependent breast cancer. GPCR30 is structurally unrelated to the family of GPCR-like membrane progesterin receptors. The identification of this distinct class of GPCR-like steroid membrane receptors suggests a widespread role for GPCRs in non-classical steroid hormone actions [61].

Several of environmental estrogens have been shown to bind to GPCR30 with the binding affinities similar to those to ERs. They activated alternative estrogen signaling pathways in an ER α -negative cell line (HEK293) stably transfected with GPCR-30. Environmental estrogens with relatively high binding affinities for GPCR30 also displayed estrogen agonist activities in an in vitro assay of membrane-bound adenylyl cyclase activity, a GPCR30-dependent signaling pathway activated by estrogens. These results indicate that nontraditional estrogen actions mediated through GPCR30 are potentially susceptible to disruption by a variety of environmental estrogens [54].

Albanito et al. [55] investigated whether ER α contributed to GPCR30/EGFR signaling. They showed that, in ER α -positive BG-1 ovarian cancer cells, both E₂ and the GPCR30-selective ligand G-1 induced c-fos expression and estrogen-responsive element (ERE)-independent activity of a c-fos reporter gene, whereas only E₂ stimulated an ERE-responsive reporter gene, indicating that GPCR30 signaling does not activate ER α -mediated transcription. Similarly, both ligands up-regulated cyclin D1, cyclin E, and cyclin A, whereas only E₂ enhanced PR expression. Moreover, both GPCR30 and ER α expression were required for c-fos stimulation and extracellular signal-regulated kinase (ERK) activation in response to either E₂ or G-1. Blockage of the EGFR transduction pathway inhibited c-fos stimulation and ERK activation by either ligand, suggesting that in ovarian cancer cells GPCR30/EGFR signaling relays on ER α expression. Interestingly, they showed that both GPCR30 and ER α expression along with active EGFR signaling were required for E₂- and G-1-stimulated proliferation of ovarian cancer cells. Because G-1 was able to induce both c-fos expression and proliferation in the ER α -negative/GPCR30-positive SKBR3 breast cancer cells, the requirement for ER α expression in GPCR30/EGFR signaling may depend on the specific cellular context of different tumor types [62].

Using the model system of SkBr3 and BT20 breast cancer cells lacking the classical ER, Albanito et al. [63] studied the regulation of GPCR30 expression by E₂, the selective GPCR30 ligand G-1, IGF-I, and EGF. Transient transfection with an expression plasmid encoding a short 5'-flanking sequence of GPCR30 gene revealed that an activator protein -1 (AP-1) site located within this region was required for the activating potential exhibited only by EGF. EGF enhanced GPCR30 protein levels, which accumulated predominantly in the intracellular compartment. The stimulatory role elicited by EGF on GPCR30 expression was triggered through rapid ERK phosphorylation and c-fos induction, which was strongly recruited to the AP-1 site. Of note, activation of the EGFR-MAPK transduction pathway by EGF stimulated a regulatory loop that subsequently engaged estrogen through GPCR30 to boost the proliferation of SkBr3 and BT20 breast tumor cells. The up-regulation of GPCR30 by ligand-activated EGFR-MAPK signaling provides new insight into the cross talk between E₂ and EGF, which contributes to breast cancer progression. The molecular details surrounding GPCR30-mediated release of proHB-EGF, the involvement of integrin β 1 as a signaling intermediary in estrogen-dependent EGFR action, and the possible implications in breast cancer progression have been described elsewhere [49,57].

3.2. Therapeutic strategies targeting GPCR-30/EGFR Signaling Pathways in TN-Breast Cancer Cells

EGFR was frequently over-expressed in TNBC [64]. The expression of GPCR30 was induced by progesterone [65] and its action was activated by E2 in breast cancer cells [66]. The plasma membrane bound GPCR30 was associated with breast tumor metastasis and transactivation of the EGFR [57]. Furthermore, EGF induced the expression of GPCR30 in TNBC [63]. It is likely that the GPCR-30/EGFR signaling pathways are important in mediating E2 effects in TNBC. Thus, EGFR-targeted therapeutic strategies in combination with radiotherapy [67,68] have potential benefits for this subgroup of breast carcinomas. Two anti-EGFR therapies with the clinical applications are monoclonal antibodies that block the binding of ligands to EGFR [69,70] and small-molecule tyrosine kinase inhibitors that inhibit the binding of adenosine triphosphate to the internal tyrosine kinase receptor of EGFR [71]. Several molecules have been synthesized to inhibit the extracellular domain of EGFR, such as cetuximab (Erbix), the extracellular domain of HER2, such as trastuzumab (Herceptin) or the EGFR tyrosine kinase domain, such as gefitinib (Iressa) and erlotinib (Tarceva)[72]. Anti-EGFR therapies have been applied in clinical trials for head, neck, lung and other cancers including breast cancer [71–75]. In addition, micro-RNAs (miRNAs) approaches have been tested for inhibition of EGFR-signaling pathways [76,77]. The miRNAs are non-coding RNAs that inhibit expression of numerous target genes. Several miRNAs have been shown to act as oncogenes or tumor suppressors. Aberrant expression and function of miRNAs have been associated with tumorigenesis. The human EGFR mRNA 3' untranslated region contains three putative microRNA-7 (miRNA-7) target sites. Webster et al. [77] observed that miRNA-7 down-regulated EGFR expression in breast, lung and glioblastoma cancer cell lines via two of three sites, inducing cell cycle arrest and cell death. Moreover, miRNA-7 also down-regulated Raf1 and several genes involved in EGFR signaling and tumorigenesis. Furthermore, miRNA-7 attenuated activation of protein kinase B (Akt) and extracellular signal-regulated kinase 1/2 (ERK1/2), two critical effectors of EGFR signaling in different cancer cell lines. It has also been shown [76] that microRNA-7 was capable of effectively inhibiting EGFR pathways in glioblastoma. These data have established an important role for miRNA-7 in controlling EGFR mRNA expression and have indicated that miRNA-7 has the ability to coordinately regulate EGFR signaling in multiple human cancer breast cancer cells, particularly in TNBC and glioblastoma [76,77]. Non-peptide ligands that target peptide-activated GPCRs have been developed for treatment of inflammation and obesity [78,79]. It is likely that development of antagonists selective for GPCR-30 deserves a great potential as therapeutic targets in TNBC. Such GPCR-30 selective antagonists in combination with anti-EGFR therapies could be potentially promising approaches for the treatment of TNBC.

4. Interactions of ER β with BRCA1/BRCA2 in TN Breast Cancer Cells

There is evidence from clinical and experimental data that familial breast cancers, including BRCA1- and BRCA2-related forms, are estrogen-sensitive [80,81]. Several studies [81–84] have reported the links between E₂/ER α and BRCA1/2. Estrogens have been shown to induce BRCA1 expression in experimental models [81]; 2) BRCA-1 has been shown to be capable of interacting with ER α via the domains within its amino- and carboxyl-termini and inhibiting ER α 's transcriptional activity in human breast. Mutations of BRCA1 abolished or reduced its ability to inhibit ER α activity in regulating estrogen-induced gene expression [82]; 3) Clinical studies have shown that endogenous or exogenous estrogens increased the risk of breast cancer in BRCA1 mutation carriers and 4) prophylactic oophorectomy decreased the risk of breast carcinoma in BRCA1/BRCA2 mutation carriers [83]. Moreover, adjuvant tamoxifen therapy

for primary breast carcinoma diminished the risk of a second breast malignancy in BRCA1 mutation carriers [84].

BRCA2 is also closely related to the pathogenesis of breast cancer. Jin et al. [85] observed that estrogen activated BRCA2 transcription via an ER α -dependent manner. During estrogen treatment, ER α interacted with co-activators CBP/p300, p68/p72, MyoD and formed an activating transcriptional complex which can bind to Sp1 sites on the BRCA2 promoter and activated its transcription by inducing histone acetylations; MyoD is a component of ER α complex; ER α or P53 attenuated ER α -mediated transcriptional activation by preventing the recruitment of ER α transcriptional complex and histone acetylations on the BRCA2 promoter; ER α interacted with ER α , CBP/p300 and formed a weak activating transcriptional complex which competed for binding to Sp1 sites with ER α transcriptional complex and slightly attenuated BRCA2 transcription; Different from ER α , p53 interacted with HDAC1 and CtBP1 and formed an inhibiting transcriptional complex which can compete for binding to Sp1 sites with ER α transcriptional complex and inhibited BRCA2 transcription more significantly.

While BRCA-1 and BRCA-2 are able to interact with ER α and regulate its transcriptional activities, a large fraction of BRCA-associated breast cancer patients are negative to ER α but express significant levels of ER β [46,80]. Thus, the effects of estrogens in these breast cancer patients might be largely mediated via ER β . It has been shown [82] that ER β replaced wild-type BRCA1 protein in controlling the proliferative response after estrogen exposure. The prevalent expression of ER β protein in BRCA1-associated tumors may explain the anti-estrogen protective effect. In fact, ER β would favor tamoxifen action by direct binding, even in the absence of ER α , and by restoring the ability of cells (impaired for modulation of strong estrogenic exposure because BRCA1 mutated) to cope with stimulation by estrogens. The interactions between ER β and BRCA1/BRCA2 and their pathological implications in TNBC remain to be investigated.

Because BRCA1 mutations, which predispose women to breast cancer, are prevalent in TNBC, nutritional and pharmacological approaches targeting the association between BRCA1 and ER β could be valuable for treatment of this group of breast cancer. Epidemiological studies have revealed that amounts of consumption of soy were inversely related to breast cancer incidence [86,87]. For example, genistein, a predominant isoflavone in soy that binds to ER β [88], has been reported to reduce the incidence of breast cancer in animal models. It inhibited BRCA1 mutant tumor growth through activation of DNA damage checkpoints, cell cycle arrest, and mitotic catastrophe [89]. Importantly, it was recently reported [90] that genistein potently inhibited growth of BRCA1 mutant cells whereas it only had a weak effect in cells expressing wild-type BRCA1 protein. The hypersensitivity of BRCA1 mutant cells to genistein could be linked to the higher expression of ER β in these cells, suggesting that phytoestrogens such as genistein could be used as efficient inhibitors of cancer development in BRCA1 mutant breast cancer cells that express ER β .

5. Important Pathological Role of CXC Chemokines in TN-breast Cancer Cells

5.1. Altered Expression of CXC Chemokines in TN-breast Cancer Cells

Chemokines (chemotactic cytokines) are a large family of cytokines that have a wide variety of biological activities. Originally, they were identified as controllers of the routine transport of immune cells by directing the migration of cells during inflammatory response. Chemokines are classified into CXC, CC, C and CX₃C subgroups on the basis of a cysteine motif near the amino terminus. The prototype chemokine, IL-8, has two cysteines (C) separated by a single amino acid (X), leading to a CXC motif. IL-8 is a CXC chemokine defined as CXCL8. Monocyte chemotactic protein-1 has two cysteines adjacent to each other (CC); members of this family are therefore known as CC chemokines. Other configurations of the disulphide

bonds have since been described, presenting a C and a CX₃C motif. The majority of chemokines are CXC chemokines. This CXC subfamily is further sub-classified into two groups, depending on the presence or absence of a tripeptide motif glutamic acid-leucine-arginine (ELR) in the N-terminal domain: ELR positive (ELR+) and ELR negative (ELR-)[91,92]. Generally, the ELR (+) CXC chemokines including CXCL8 and related CXCL1, CXCL2 and CXCL3 [also called growth-related oncogene α , β and γ (GRO α , GRO β and GRO γ), respectively], CXCL4, CXCL4V1, CXCL5, CXCL6, and CXCL7 that bind to CXCR2 are angiogenic whereas ELR (-) CXC chemokines, including CXCL9 (Mig), CXCL10 (IP-10), CXCL11 (I-TAC), that bind to CXCR3 are angiostatic. An exception ELR (-) CXC chemokine is stromal cell-derived factor-1 (CXCL12/SDF-1), which binds to both CXCR4 and CXCR7, is angiogenic, and implicated in tumor metastasis [92].

Recent studies have indicated that several CXC chemokines are the major players in breast cancer. CXCL8 (IL-8) is a multi-functional chemokine that has important biological functions in tumor formation and development. Freund et al. [93] reported that CXCL8 was over-expressed in most ER α -negative breast cell lines and breast tumor samples, whereas no significant IL-8 levels were detected in ER α -positive breast cell lines. Human CXCL8 from ER α -negative MDA-MB-231 cells and breast cancer cells was identical to monocyte-derived CXCL8. The invasion potential of ER α -negative breast cancer cells was shown to be associated at least in part with over-expression of CXCL-8, but not with CXCL-8 receptor (CXCR2) levels. Moreover, CXCL-8 also increased the invasiveness of ER-positive breast cancer cells by two fold. On the other hand, forced expression of ER α in ER α -negative cells led to a decrease in CXCL8 levels. These data indicate that IL-8 expression is negatively linked to ER α status of breast cancer cells and that IL-8 expression is associated with a higher invasiveness potential of cancer cells.

Lin et al. [94] have identified CXCL8 as a key factor involved in breast cancer invasion and angiogenesis. They observed elevated expression of CXCL-8 in breast cancer cells. Neutralization of CXCL-8 with CXCL-8 antibody specifically blocked CXCL-8-mediated tumor cell invasion and angiogenesis. Furthermore, CXCL-8 levels in human breast cancer cells were inversely related to ER status, i.e. ER positive breast cells expressed low levels of CXCL-8 whereas ER α negative cells expressed high levels of CXCL-8. Expression of exogenous ER α substantially inhibited CXCL-8 expression. The angiogenic effect of CXCL-8 in breast cancer cells and its association with ER α status was confirmed by another study by Lin et al. [95]. These researchers collected the culture supernatants of breast cancer cells with high expression of CXCL-8 from MDA-MB-231 and MDA-MB-157, moderate expression of CXCL-8 from SKBr-3, and low expression of CXCL-8 from T47D and ZR75-1 cells, and examined the effects of these supernatants on cell migration of human umbilical cord vein endothelial cells. They found that the numbers of migrating HUVECs cultured in the supernatants of MDA-MB-231 cells, SKBr-3 cells, and T47D cells were progressively reduced from 7800 to 6510 to 3470, respectively whereas addition of CXCL-8 neutralizing antibody significantly reduced the number of migrating HUVECs cultured in the supernatant of MDA-MB-231 cells. The HUVECs cultured in the supernatants of the breast cancer cells expressing higher level of IL-8 tended to form more microangioid structure and proliferated more rapidly than those cultured in the supernatants of the breast cancer cells expressing lower level of IL-8. The skin of the mice subcutaneously injected with the supernatants of the breast cancer cells expressing higher level of IL-8 plus fibroblast growth factor (FGF)-2 for five days formed more blood vessels. Transient co-transfection with ER α and pN1481 Luc containing IL-8 promoter or p Luc0 not containing IL-8 promoter showed that the CXCL-8 level of the MDA-MB-231 cells transfected with ER α was decreased by 8.8 folds compared with that of the ER α -negative MDA-MB-231 cells. The activity of CXCL-8 promoter was significantly reduced by the expression of ER α . These results reveal that CXCL-8 is a key factor involved in angiogenesis of human breast cancer cell and that the CXCL-8 level in human breast cancer

cells is negatively correlated with ER α status. Yao et al. [96,97] observed that knock down of CXCL-8 expression with specific siRNA in ER α -negative MDA-MB-231 and MDA-MB-468 cells significantly reduced cell invasion but had no effects on cell proliferation and cell cycle. In vivo suppression of CXCL-8 led to significant reduction in the micro-vessel density and marked reduction in neutrophil infiltration into the tumors.

While CXCL-8 is over-expressed in ER α -negative breast cell lines, an analysis of CXCL-8 chromosomal location [85] has also revealed that several CXCL8-related CXC chemokines, including CXCL1, CXCL2, CXCL3, CXCL4, CXCL4V1, CXCL5, CXCL6, and CXCL7, are all localized in the same narrow region of chromosome 4 and thus, belong to the same cluster. Quantitative measurement of these chemokines in breast tumors [98] has revealed that the breast cancer samples that expressed low levels of ER α but high levels of CXCL8 produced increased levels of CXCL1, CXCL3, and CXCL5. Moreover, CXCL1, CXCL2, CXCL3, CXCL5, and CXCL8 were co-regulated in both tumors and breast cancer cell lines. CXCL5 and CXCL8 were mainly expressed in epithelial cells whereas CXCL1, CXCL2, and CXCL3 were highly expressed in blood cells. The over-expression of these chemokines in tumor cells was not the result of gene amplification but rather of an enhanced gene transcription. Furthermore, high CXCL8 expression in tumors was mainly correlated to AP-1 pathway and to a minor extent to NF- κ B pathway. Interestingly, higher levels of CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8 were present in metastatic breast cancer when compared with grade I and III biopsies. High levels of CXCL8, CXCL1, and CXCL3 accounted for a shorter relapse-free survival of ER α -positive patients treated with TAM.

Vazquez-Martin et al. [99,100] examined the profile of cytokines in conditioned media obtained from MCF-7/HER2-18 cells, a MCF-7-derived clone engineered to stably express the full-length human HER2 cDNA, and from the MCF-7/neo control sub-line using Human Cytokine Array III. They observed and confirmed at least 10-fold increase in CXCL8 and CXCL1 levels in MCF-7/HER2 cells as compared to those MCF-7/neo control sub-line. Treatment with the tyrosine kinase inhibitor gefitinib returned the expression levels of IL-8 and CXCL-1 back to the baseline observed in HER2-negative MCF-7 BC cells. Moreover, the circulating levels of CXCL8 and CXCL1 were significantly higher in sera from HER2-positive breast cancer patients. It has been also observed [88] that EGF potently up-regulated CXCL8 secretion by breast tumor cells, and this effect was promoted by a consecutive treatment of the cells with estrogen and progesterone. Given that EGFR is over-expressed and ER β is present in TNBC cells, there could be combined, synergetic effects of EGF and E₂ via GPCR-30/EGFR and, perhaps, ER β -mediated pathways on the levels of CXCL8 and its related CXC chemokines. Tang et al. [101] reported that the expression of CXCL7, a heparin binding ligand that has heparinase activity, and its receptor, CXCR2, was higher in malignant MCF10CA1a.cl1 cells than in pre-malignant MCF10AT cells that do not express ER α and that transfection of CXCL7 into pre-malignant MCF10AT resulted in increased invasion through basement membrane.

Together, these lines of evidence indicate that multiple CXC chemokines are co-expressed in ER α -negative breast tumors and in breast tumors expressing HER-2. These CXC-chemokines could account for the higher invasiveness and aggressiveness of these types of breast tumors. CXCL8 and its related CXC chemokines could be the novel markers of tumor aggressiveness in TNBC.

5.2. Targeting CXCL8 and Related Chemokines in TN-breast Cancer Cells

Over-expression of CXCL8 and its related CXC chemokines in ER α -negative and TNBC patients has pointed to involvement of these chemokines and their respective receptors in progression and metastasis of these subgroups of breast cancer. It is important to determine the precise roles of these chemokines in pathogenesis in TNBC and their regulation by E₂/

ER β in TNBC. The circulating levels of CXCL8 and related GRO chemokines represent novel biomarkers for monitoring TNBC in response to endocrine treatments and/or HER2-targeted therapies.

Targeting these chemokines and their respective receptors by antibodies, small molecule antagonists, viral chemokine binding proteins and heparins appears to be promising tracks to develop therapeutic strategies. There is significant interest in developing strategies to antagonize the chemokine function, and an opportunity to interfere with metastasis which causes death in most patients [102]. An approach has been reported for specifically targeting CXCL-8 and its receptor, CXCR2, using repertaxin, a non-competitive blocker of CXCR1 and CXCR2. Repertaxin acts by locking CXCR1/CXCR2 in an inactive conformation, preventing receptor signaling and human polymorphonuclear leukocyte chemotaxis. It has been shown that repertaxin potently and selectively blocked PMN adhesion to fibrinogen and CD11b up-regulation induced by CXCL8. Reduction of CXCL8-mediated PMN adhesion by repertaxin was paralleled by inhibition of PMN activation including secondary and tertiary granule release and pro-inflammatory cytokine production. Repertaxin also selectively blocked CXCL8-induced T-lymphocyte and natural killer cell migration. These data suggest that repertaxin is a potent and specific inhibitor of a wide range of CXCL8-mediated activities related to leukocyte recruitment and functional activation in inflammatory sites [103].

6. MicroRNA Signatures, Suppression of ER α Expression and ER α -Signaling by micro-RNAs in ER α -negative Breast Cancer Cells

MicroRNAs (miRNAs) are a class of small non-coding RNAs that control gene expression by targeting mRNAs and triggering either translation repression or RNA degradation. MiRNAs have emerged as important regulators of gene expression in a plethora of physiological and pathological processes. However, they have been found to be aberrantly expressed in breast cancer cells, where they function as regulators of tumor behavior and progression [104].

The miRNA signatures predict ER, PR and HER2 status in breast cancer [105,106]. For example, Lowery et al. [105] examined the expression profile of 453 miRNAs in 29 early stage breast cancer specimens. Using stepwise artificial neural network analysis, these investigators identified predictive miRNA signatures corresponding with the status of ER α (miR-342, miR-299, miR-217, miR-190, miR-135b, miR-218), PR (miR-520g, miR-377, miR-527-518a, miR-520f-520c) and HER2 (miR-520d, miR-181c, miR-302c, miR-376b, miR-30e). Further analysis of miR-342 and miR-520g in 95 breast tumors revealed that miRNA-342 expression was highest in ER α and HER2 positive luminal B tumors and lowest in TNBC tumors whereas miRNA-520g expression was elevated in ER α - and PR- negative tumors. The association of specific miRNAs with ER, PR and HER2 status indicates a role for these miRNAs in disease classification of breast cancer. Decreased expression of miRNA-342 in TNBC tumors, increased miRNA-342 expression in the luminal B tumors, and down-regulated miRNA-520g in ER- and PR- positive tumors indicates that deregulated miRNA expression is not only a marker for poorer prognosis of breast cancer, but it could also present an attractive target for therapeutic intervention [105].

Recent studies [106–112] have shown that a number of miRNAs are involved in regulation of ER α expression and ER α -mediated signaling in breast cancer cells. The expression levels of ER α in breast cancer tissues differ widely among patients, and frequently change during disease progression and in response to systemic therapies. While multiple mechanisms involved in altering ER α gene expression in breast cancer, including ER α gene amplification, transcriptional silencing by DNA methylation of CpG islands within the ER α promoter and mutations within the open reading frame of ER α , have been identified (for review see [109]), miRNAs are emerging as important regulators in controlling ER α expression and functions in

breast cancer cells. The ER α mRNA has a about 4.3 kb 3'-untranslated region (3' UTR), which has been reported to reduce mRNA stability and which bears evolutionary conserved miRNA target sites, suggesting that ER α might be regulated by miRNAs [113]. Several miRNAs, including miR-221/222 [106,111], miR-206 [107,108], miR-18a [110], and miR-22 [113], have been shown to be involved in down regulation of ER α expression and in suppression of ER α -mediated signaling in breast cancer cells. They are described as follows.

In a search for regulators of ER α expression, Zhao et al. [106] have identified a set of miRNAs whose expression was specifically elevated in ER α -negative breast cancer cells. They observed a distinct expression of a panel of miRNAs between ER α -positive and ER α -negative breast cancer cell lines and primary breast tumors. Of the elevated miRNAs in ER α -negative cells, miR-221 and miR-222 were found to directly interact with the 3'-UTR of ER α . Ectopic expression of both miRNAs in MCF-7 and T47D cells resulted in a decreased expression of ER α protein but not its mRNA, whereas knockdown of their expression partially restored ER α in ER α protein-negative/mRNA-positive cells. More importantly, transfection of MCF-7 and T47D cells with miRNA-221- and/or miRNA-222-containing vectors caused these cells to become resistant to tamoxifen compared with vector-treated cells whereas knocking down their expression in MDA-MB-468 cells sensitized these cells to tamoxifen-induced cell growth arrest and apoptosis. Consistent with these observations, Miller et al. [111] reported that miR-221 and miR-222 confer tamoxifen resistance in breast cancer by targeting p27Kip1, a cell cycle inhibitor. These findings indicate that miR-221 and miR-222 play a significant role in the regulation of ER α expression at the protein level and could be potential targets for restoring ER α expression and responding to anti-estrogen therapy in a subset of ER α -negative breast cancers.

The expression of another miRNA, namely miR-206, has been shown to be higher in ER α -negative breast cancer MB-MDA-231 cells than in ER α -positive MCF-7 cells. This miRNA targets the human ER α , repressing its mRNA and protein expression in breast cancer cell lines [108]. Furthermore, its expression was markedly decreased in ER α -positive human breast cancer tissues and was inversely correlated with ER α but not ER β mRNA expression in breast cancer tissues [109]. Two miR-206 binding sites (hER α 1 and hER α 2) have been identified in 3'-UTR of ER α . These binding sites have been shown to respond accordingly to exogenous hsa-pre-miR-206 and 2'-O-methyl antagomiR-206. Mutations that disrupted hybridization to the 5'-seed of miR-206 inactivated these responses. A C $\rightarrow$ T single nucleotide polymorphism in the hER α 1 site increased repression of luciferase activity to approximately 3.3-fold in HeLa cells. Interestingly, its expression was strongly inhibited by ER α agonists, but not by an ER β agonist or progesterone, indicating a mutually inhibitory feedback loop [108]. Introduction of miR-206 into estrogen-dependent MCF-7 cells inhibited cell growth in a dose- and time-dependent manner [109].

More importantly, miRNA-206 has been shown to play an important role in the EGF-induced repression of ER α signaling and a Luminal-A phenotype in MCF-7 cells [107]. EGFR/MAPK signaling can induce a switch in MCF-7 cells, from an ER α -positive, Luminal-A phenotype, to an ER α -negative, basal-like phenotype. It coordinately targeted mRNAs encoding the co-activator proteins SRC-1 and SRC-3, and the transcription factor GATA-3, all of which contributed to estrogenic signaling and a Luminal-A phenotype. Over-expression of miRNA-206 repressed estrogen-mediated responses in MCF-7 cells, even in the presence of ER α encoded by an mRNA lacking a 3' UTR, suggesting miRNA-206 affects estrogen signaling by targeting mRNAs encoding ER α -associated co-regulatory proteins. EGF treatments enhanced miRNA-206 levels in MCF-7 cells and ER α -negative, EGFR-positive MDA-MB-231 cells, while EGFR siRNA, or PD153035, an EGFR inhibitor, or U0126, a MAPK kinase [114] inhibitor, significantly reduced miR-206 levels in

MDA-MB-231 cells. Blocking EGF-induced enhancement of miRNA-206 with antagomiR-206 abrogated the EGF inhibitory effect on ER α , SRC-1 and SRC-3 levels, and on ERE-luciferase activity, indicating that EGFR signaling represses estrogenic responses in MCF-7 cells by enhancing miR-206 activity. Elevated miR-206 levels in MCF-7 cells ultimately resulted in reduced cell proliferation, enhanced apoptosis, and reduced expression of multiple estrogen-responsive genes. These results indicate that miR-206 contributes to EGFR-mediated abrogation of estrogenic responses in MCF-7 cells, and to a Luminal-A to Basal-like phenotypic switch, and may be a measure of EGFR-response within basal-like breast tumors.

Two additional miRNAs, namely miRNA-18a [110] and miR-22 [113], have been shown to inhibit estrogen signaling by directly targeting the ER α mRNA. Liu et al. [110] observed that miRNA-18a was increased specifically in samples from female hepatocellular carcinoma cancer (HCC) patients and its increased levels in female HCC tissues were correlated with reduced ER α expression. Over-expression of miR-18a decreased ER α levels but stimulated the proliferation of hepatoma cells. In a comprehensive and systematic assessment of the regulatory role of miRNAs that were predicted to target the 3' UTR of the ER α , Pandey et al. [113] identified miR-22, which repressed strongly ER α expression by directly targeting the 3' UTR of ER α . There are three predicted miR-22 target sites in the 3' UTR, of which, the evolutionary conserved one is the primary target. Over-expression of miR-22 led to reduced ER α levels, in part by inducing mRNA degradation, and compromises estrogen signaling as exemplified by its inhibitory impact on the ER α -dependent proliferation of breast cancer cells.

On the other hand, the expression of other miRNAs could be increased in ER α -positive breast cancer cells. For example, the miR-21 has been found to be higher in ER α positive than negative tumors and its expression is reduced by E₂.

Wickramasinghe et al. [112] reported that E₂ inhibited miR-21 expression in MCF-7 cells and this effect was inhibited by ER antagonist, 4-hydroxytamoxifen (4-OHT), ICI182780, and siRNA ER α , indicating that the suppression is ER α -mediated. ER α and ER β agonists PPT and DPN inhibited and 4-OHT increased miR-21 expression. E₂ increased luciferase activity from reporters containing the miR-21 recognition elements from the 3'-UTRs of miR-21 target genes, corroborating that E₂ represses miR-21 expression resulting in a loss of target gene suppression. The E₂-mediated decrease in miR-21 correlated with increased protein expression of endogenous miR-21-targets, e.g. Pdc4, PTEN and Bcl-2. Knockdown of ER α with its siRNA blocked the E₂-induced increase in Pdc4, PTEN and Bcl-2. Transfection of MCF-7 cells with antisense to miR-21 mimicked the E₂-induced increase in Pdc4, PTEN and Bcl-2. These results demonstrate that E₂ represses the expression of an oncogenic miRNA, miR-21, by activating ERs in MCF-7 cells.

Together, these findings have provided lines of the strong evidence for the transcriptional and posttranscriptional regulation of ER α by a group of miRNAs including miRNA-221/222 [106,111], miRNA-206 [107,108], miRNA-18a [110], and miRNA-22 [113] in breast cancer. The up-regulation of these miRNAs and their involvement in down-regulation of ER α expression and thus, suppression of ER α -signaling pathways have provided a clear explanation for one of the major causes of ER α -negative status and the insensitiveness of endocrine therapy in a fraction of breast cancer cells including TNBC cells. These findings also indicate that these miRNAs could be explored as novel candidates for endocrine therapy that targets only ER α in breast cancer cells. By regulating the signatures of these miRNAs, it is likely to find a better way for treating ER α -negative BLBC.

7. Altered Expression of Several oncogenic proteins and tumor suppressors in TN-breast Cancer Cells

Altered expression of a number of oncogenic and tumor suppressor proteins and abnormal signaling pathways have been detected in TN-breast cancer cells. They are described below.

7.1. Altered Notch-Survivin Signaling in ER-negative breast cancer cells

Notch-ligand interaction is a highly conserved mechanism that regulates specific cell fate decision during cell development and maturation. However, accumulating pre-clinical and clinical evidence indicates that Notch signaling functions as a pro-oncogene in several solid tumors, particularly in breast cancer. Notch activation plays a role in the onset and progression of many human malignancies [115–117]. It has been shown that Notch-1 was over-expressed in breast cancer and that high expression of Notch-1 and Jagged-1 correlated with poor prognosis and progressively abbreviated overall survival, and associated with increased expression of survivin, a tumor-associated cell death and mitotic regulator implicated in stem cell viability [118,119]. Notch-1 and survivin co-segregated in basal breast cancer. Notch-1 stimulation in MDA-MB-231 cells increased survivin expression, whereas silence of Notch expression reduced survivin levels [119]. Lee et al. [119] have shown that activation of Notch signaling in ER α -negative breast cancer cells resulted in direct transcriptional up-regulation of the apoptosis inhibitor and cell cycle regulator survivin. This response was associated with increased expression of survivin at mitosis, enhanced cell proliferation, and heightened viability at cell division. Rizzo et al. [118] examined expression of Notch receptors and ligands in clinical specimens, as well as activity, regulation, and effectors of Notch signaling using cell lines and xenografts. They observed that ductal and lobular carcinomas commonly expressed Notch-1, Notch-4, and Jagged-1 at variable levels. However, in breast cancer cell lines, Notch-induced transcriptional activity was highest in ER α -negative, Her2/Neu overexpressing cells but did not correlate with Notch receptor levels. In ER α -positive cells, E2 inhibited Notch activity and Notch-1 nuclear levels, and affected Notch-1 cellular distribution. Tamoxifen and raloxifene blocked these effects, reactivating Notch. Notch-1 induced Notch-4. Notch-4 expression in clinical specimens correlated with proliferation marker, Ki67. In MDA-MB231 (ER α -negative) cells, Notch-1 knockdown decreased cyclins A and B1, causing G [120] arrest, p53-independent induction of NOXA, and death. In T47D: A18 (ER α -positive) cells, the same targets were affected, and Notch inhibition potentiated the effects of tamoxifen. These observations indicate that a Notch-1/survivin gene signature is a hallmark of BLBC, which may contribute to pathogenesis of TNBC [119,121].

There is evidence [122,123] that notch pathway play a role in estrogen-induced angiogenesis in breast cancer cells. Angiogenesis is the formation of blood vessels based on preexisting one and is an important process of tumorigenesis. E₂ has been shown to promote expression of Jagged1 expression and Notch1 expression in MCF7 cells and endothelial cells. These effects were abrogated by an estrogen antagonist, ICI182780. Consistent with these effects of E₂, imperfect estrogen-responsive elements were found in the 5' untranslated region of Notch1 and Jagged1 genes. E₂ treatment activated Notch signaling in MCF7 cells expressing Notch1 reporter gene or promoted Jagged1-induced Notch signaling in coculture assays. Inoculation of MCF7 cells in E₂-treated nude mice resulted in up-regulation of Notch1 expression as well as increased number of tumor microvessels in comparison to placebo-treated mice. Notch1-expressing endothelial cells formed cordlike structures on Matrigel whereas cells expressing a dominant-negative form of Notch1 did not form this structure, emphasizing the relevance of Notch1 pathway in vessel assembly. Furthermore, Notch1-expressing MCF7 cells up-regulated hypoxia-inducible factor

1 alpha gene, a well-known angiogenic factor that clustered with Notch1 gene. These studies implicate Notch signaling in the cross talk between E₂ and angiogenesis.

Inhibition of Notch-Survivin pathway by using gamma-secretase inhibitors can be a novel molecular therapy for recurrence-prone breast cancer patients. Gamma-secretase, a large membrane-integral multi-subunit protease complex, is essential for Notch receptor activation. It has been shown [121] that targeting Notch signaling with a peptidyl gamma-secretase inhibitor suppressed survivin levels, induced apoptosis, abolished colony formation in soft agar, and inhibited localized and metastatic tumor growth in mice, without organ or systemic toxicity. In contrast, ER positive breast cancer cells, or various normal cell types, were insensitive to Notch stimulation. ER α -negative breast cancer cells became dependent on Notch-survivin signaling for their maintenance, in vivo. Recently, Gamma-secretase inhibitors, as well as various biopharmaceutical or genetic Notch signaling inhibitors have been suggested as potential novel cancer therapeutic strategies. Therapeutic target of this pathway may be explored for individualized treatment of patients with clinically aggressive, ER α -negative breast cancer. For example, it has been shown that in vivo, gamma-secretase inhibitor treatment arrested the growth of ER α -negative MDA-MB231 tumors whereas this inhibitor in combination with tamoxifen caused regression of ER α -positive T47D: A18 tumors. These data indicate that combinations of anti-estrogens and Notch inhibitors can be effective in ER α -positive breast cancers whereas Notch signaling can be a potential therapeutic target in ER α -negative breast cancers [118,124].

7.2. AKT Activation by Estrogens in ER α -Negative Breast cancer Cells

It has been shown that in the cytoplasm, the ER-dependent signaling pathways are involved in the activation of Akt and the downstream molecules. Tsai et al. [125] determined whether estrogen modulated cytoplasmic signaling in an ER-independent manner. They treated MDA-MB-435 and MDA-MB-231 with estrogen and observed that estrogen stimulated Akt activation, as indicated by phosphorylation at Ser (473) of the oncoprotein, in these ER α -negative breast cancer cells. Activation of Akt by estrogen in these cells was time- and dose-dependent, which was blocked by inhibitors of phosphatidylinositol 3'-protein kinase (PDK-1) and Src kinase but not by estrogen antagonists.

Weng et al [126] used a small-molecule inhibitor of PDK-1, OSU-03012, to determine whether PDK-1/Akt signaling represented a therapeutic target to sensitize ER-negative breast cancer to tamoxifen. OSU-03012 sensitized both ER-positive MCF-7 and ER-negative MDA-MB-231 cells to the anti-proliferative effects of tamoxifen in an ER-independent manner by a marked enhancement of tamoxifen-induced apoptosis. This OSU-03012-mediated sensitization was associated with suppression of a transient tamoxifen-induced elevation of Akt phosphorylation and enhanced modulation of the functional status of multiple Akt downstream effectors, including FOXO3a, GSK3 α/β , and p27. The growth of established MDA-MB-231 tumor xenografts was suppressed by 50% after oral treatment with the combination of tamoxifen (60 mg/kg) and OSU-03012, whereas OSU-03012 and tamoxifen alone suppressed growth by 30% and 0%, respectively. These findings indicate that the inhibition of PDK-1/Akt signaling to sensitize ER-negative breast cancer cells to the ER-independent antitumor activities of tamoxifen represents a feasible approach to extending the use of tamoxifen to a broader population of breast cancer patients including TN-breast cancer patients.

7.3. Altered Expression of E-Cadherin and E-selectin in TN-Breast cancer Cells

As mentioned above, angiogenesis plays an important role in breast cancer growth and metastasis. Multiple adhesion molecules including E-cadherin and E-selectin have been shown to play critical roles in angiogenesis.

E-cadherin, a calcium-dependent epithelial cell adhesion molecule, has been implicated as a tumor suppressor [109]. E-cadherin inactivation in breast cancer was strongly associated with lobular breast cancer. Mahler-Araujo et al. [127] performed immunohistochemical analysis for E-cadherin expression and distribution in a tissue microarray containing duplicate cores of 245 invasive breast carcinomas, of which 182 cases were of non-lobular histology. They observed that in non-lobular breast carcinomas, a reduced and/or negative E-cadherin expression was significantly associated with the lack of ER expression, low levels of CCND1 expression, positivity for cytokeratins 5/6 and 17, EGFR and caveolins 1 and 2, p53 expression, high MIB-1 proliferation indices, basal-like phenotype and TN-phenotype. This study demonstrated that in the group of non-lobular breast cancers, reduction or lack of E-cadherin expression was preferentially found in BLBC. As E-Cadherin was regulated by estrogens [128], it is possible that estrogens promotes breast cancer by down-regulation of E-cadherin levels [129]. Down-regulation of E-cadherin in human bronchial epithelial cells led to EGFR-dependent Th2 cell-promoting activity [130].

Nguyen et al. [131] analyzed 15 benign and 22 malignant ER α -negative and ER α -positive breast specimens for the presence of E-selectin and P-selectin. They found that E-selectin's expression was increased in the malignant breast tumors compared with their benign counterparts. Furthermore, E-selectin staining was significantly increased in the ER α -negative carcinomas compared with the ER α -positive ones. This in vitro findings strongly correlated with the in vivo findings and showed a higher degree of E-selectin induction in endothelial cells exposed to conditioned media from ER α -negative breast cancer cell lines than from ER α -positive ones. The degree of E-selectin induction was correlated with the amount of interleukin-1 α in the tumor-conditioned media. Neutralizing antibodies to interleukin-1 α significantly inhibited the E-selectin expression in endothelial cells exposed to tumor-conditioned media. The results indicate that the endothelial E-selectin expression during angiogenesis is related to breast carcinoma progression in vivo and that this component of angiogenesis may be due to tumor-cell-secreted interleukin-1 α .

7.4. Altered Expression of Osteopontin in TN-breast Cancer

Osteopontin is a chemokine-like phosphorylated glycoprotein that plays important role in cancer progression and is found to be a metastasis-associated protein in breast cancer. Wang et al. [132] evaluated osteopontin protein expression in TNBC to determine if they correlated with clinicopathological parameters on 117 breast carcinoma tissue samples, and then assessed the mean value of osteopontin expression against TN-status and clinicopathological parameters. Of the 239 patients in the study, 47 were classified as triple negative. Of the 117 osteopontin-test patients in this cohort, mean osteopontin levels were significantly higher in the TNBC than in non-TN subtype. Tumours, nodes, metastases stage was significantly associated with osteopontin levels. Univariate analysis has showed that tumor cell osteopontin positivity is significantly associated with decreased disease-free survival, but not with overall survival. In the multivariate model, osteopontin was an independent prognostic factor for disease-free survival. This study has revealed that patients with osteopontin over-expression develop predominantly TNBC and that osteopontin over-expression is associated with tumor aggressiveness and poor prognosis.

8. Importance of Genotoxic Effects Caused by Oxidative Estrogen Metabolites in TN-Breast Cancer Cells

Oxidative metabolism of E₂ into the catecholestrogens (CEs), e.g. 4-hydroxyestradiol (4-OHE₂) and 2-OHE₂ which can be oxidized to the corresponding ortho-quinone derivatives with concomitant formation of the reactive oxygen species (ROS), have been thought to play an important role in estrogen carcinogenesis in breast cancer [6]. It has been demonstrated

[133–135] that E₂, 4-OHE₂ and 2-OHE₂ were capable of transforming immortalized human breast epithelial cells, MCF-10F cells that do not express the phenotypes of neoplastic transformation. The transformed MCF-10F cells formed colonies in agar-methocel, exhibited increased chemotaxis, chemoinvasion and tumorigenic in SCID mice. It is important to note that MCF-10F cells share several properties with TN-breast epithelial cells in that ER α and PR are undetectable in these cells [134–137] and that these cells contain substantial amounts of EGFR [138] and expressed ER β [136,137,139]. Thus, MFC-10F cells could serve as an experimental model of TN-breast epithelial cells.

Several lines of evidence have strongly pointed to an important contribution of the genotoxic effects caused by CEs to E₂-mediated transformation and tumorigenesis in MCF-10F cells. First, E₂-mediated transformation and tumorigenesis of MCF-10F cells were associated with loss of chromosome 4 and 8p11.21–23.1, deletions in chromosomes 3p12.3–13, 8p11.1–21, 9p21-qter, and 18q, and gains in 1p, and 5q15-qter as well as chromosomal amplifications in 1p36.12-pter, 5q21.1-qter, and 13q21.31-qter [133,140]; Second, it has been demonstrated [141,142] that active estrogen metabolism and formation of estrogen-DNA adducts were present in MCF-10F cells. Importantly, these effects were strongly induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin, a potent inducers of cytochrome P450 1B1 that catalyzes the conversion of E₂ to CEs, and were reduced by inhibition of catechol-O-methyltransferase, which catalyzes the methylation of CEs, and thereby detoxify them [141,143]; Third, it has been shown [144] that estrogen-DNA adduct formation in MCF-10F cells was prevented by resveratrol, a naturally occurring phytoalexin present in grapes and other foods that possesses chemopreventive effects.

It is likely that increased E₂ levels, oxidative E₂ metabolism and the resulting oxidative genotoxic effects are an important factor contributing to estrogen carcinogenesis in TNBC, particularly in BRCA-associated breast cancer. Aromatase is the rate-limiting enzyme in estrogen biosynthesis. Aromatase expression in ovarian granulosa cells dictated levels of circulating estrogen in pre-menopausal women, and its aberrant over-expression in breast adipose tissues promoted breast cancer growth. Aromase expression in breast cells was under the control of BRCA1 [145]. It has been known [135] that BRCA1 negatively regulated the cancer-associated aromatase promoters I.3 and II in breast adipose fibroblasts and malignant epithelial cells. In normal adipose tissue, transcription of the aromatase gene was initiated from a relatively weak adipose-specific promoter (I.4). BRCA1 inhibited a breast cancer-associated promoter of the aromatase gene in human adipose stromal cells. However, in breast cancer, a switch of promoter utilization from I.4 to a strong ovary-specific promoter, PII, led to increased aromatase expression. Ghosh et al. [146] reported an intriguing relationship between BRCA1 and aromatase expression in human adipose stromal cells (ASCs). Upon stimulation by dexamethasone, increased aromatase expression in ASCs was accompanied by a significant reduction of the BRCA1 level. In addition, adipogenesis-induced aromatase expression was also inversely correlated with BRCA1 abundance. Down-regulation of BRCA1 expression in response to various stimuli was through distinct transcription or post-transcription mechanisms. Importantly, siRNA-mediated knockdown of BRCA1 led to specific activation of the breast cancer-associated PII promoter. Therefore, BRCA1 plays a role in modulation of estrogen biosynthesis in ASCs, which may also contribute to its tissue-specific tumor suppressor function. It is likely that in BRCA1 mutation carriers or BRCA1 deficiency in epithelial and certain non-epithelial cells, the negative control of aromatase expression by BRCA1 is lost, leading to increased expression of aromatase and increased levels of E₂ which is metabolized to CEs. The change from the wild-type to the mutant BRCA1 in BRCA-1 associated breast cancer patients may be

accompanied by a “endocrine-genotoxic” switch predominantly toward a direction of DNA-damaging effects [147].

The relative contribution of CE-mediated genotoxic pathways to estrogen carcinogenesis in TNBC remains to be further evaluated. If these pathways are proved to be one of the predominant pathways in pathogenesis of TNBC, then a combination of specific inhibition of aromatase/cytochrome P450s (e. g. P450 1B1), stimulation of COMT activity and cellular anti-oxidant systems, as well as induction of oxidative DNA repair systems should be effective alternatives for targeting these genotoxic pathways in TNBC. In this regard, selected natural chemopreventive agents including N-acetylcysteine, melatonin, reduced lipoic acid, and phetostrogens e.g. resveratrol have been shown to be effective in inhibition of depurinating estrogen-DNA adduct formation and estrogen carcinogenesis [143,144].

9. Conclusions and Perspective

The present review has provided new insights into the pathogenesis of TNBC by describing several newly identified features of TNBC, as summarized in Figure 1, including: a) the existence of E₂/ER β -mediated signaling pathways; b) GRCP-30/EGFR signaling pathway; c) Interactions between ER β and BRCA1; d) CXCL8 and related CXC Chemokines; e) MicroRNA signatures and role of miRNAs in suppression of ER α expression and ER α -signaling and f) altered expression of several oncogenes and tumor suppressor-mediated signaling pathways and g) genotoxic effects caused by oxidative estrogen metabolites, in ER α -negative and TNBC. While these molecular pathways could be relevant to ER α -negative and TNBC, more studies are needed to comprehensively determine the precise and the relative contributions of each and a combination of these pathways to the pathogenesis of ER α -negative and TNBC. Such studies will be valuable not only for gaining better understanding on the pathogenesis of TNBC but also allow identification and development of novel biomarkers/targets for diagnostic and therapeutic approaches for prevention and treatment of these types of breast cancer. Of importance and relevance are: the connection of this type of tumor with the environmental estrogens linking this type of cancer with an exogenous causative agent; and the potential treatment of this type of breast cancer with naturally derived chemopreventive agents.

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Figure 1. Molecular Features of TNBC: 1)

E₂/ERβ-mediated signaling pathways and Interaction with BRCA1; 2) E₂/GRCP-30/EGFR signaling pathway; 3) CXCL8 and related CXC Chemokines; 4) MicroRNA signatures; 5) Notch/Suvivin; 6) Akt; 7) E-cadherin/E-Selectin and 8) Osteopontin are shown. Basic shapes filled with colors indicate increased expression; Basic shapes filled without color [148] indicate loss or reduced levels of the indicated proteins/receptors

Table 1

Several immunohistochemical and pathological features of breast cancer subgroups.

Properties BC Subgroups	ER, PR, Her2 Status	Other Markers	Clinopathological Features
Luminal A (LA)	ER+/PR+, Her2–	Relatively lower Ki67	Good prognosis Ductal carcinomas plus relatively higher % of lobular carcinomas Lower % of poorly differentiated carcinomas
Luminal B (LB)	ER+, PR+, HER2+	Higher FoxA1 Higher Proliferting Markers e.g. Ki67, CCNB1, MYBL2	Poorer outcomes Poor prognosis Worse patient Prognosis Smaller tumor size
Basal-Like Breast Cancer (BLBC)	ER–, PR–, HER2– CK 5/6 ⁺ and EGFR ⁺	Reduced expression of genes related to ER α Markers of myoepithelial cell Associated with Mutant BRCA1	Worse Overall & Disease free survival Poorly differentiated carcinomas Majority ductal carcinomas
HER2-enriched	High Her2 ⁺ , ER–, PR–	NA	Increased risk of breast cancer relapse & death Poorly differentiated carcinomas Majority ductal carcinomas

BRCA1: breast cancer 1; **CCNB1**: G2/mitotic-specific cyclin B1; **CK 5/6**: cytokeratin 5/6; **EGFR**: Epithelial growth factor receptor; **ER**: estrogen receptor; **FOX A1**: Forkhead box protein A1; **HER-2**: Human Epithelial growth factor receptor-2; **Ki67**: Antigen identified by monoclonal antibody; **MYBL2**: V-myb myeloblastosis viral oncogene homolog (avian)-like 2, and **PR**: Progesterone Receptor. +: represents positive; –: represents negative.

+ : Positive

– : Negative