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Critical Roles of EGFR Family Members in Breast Cancer and Breast Cancer Stem Cells: Targets for Therapy

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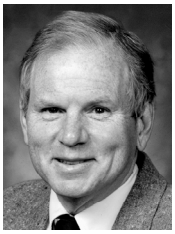

James A. McCubrey

Abstract: The roles of the epidermal growth factor receptor (EGFR) signaling pathway in various cancers including breast, bladder, brain, colorectal, esophageal, gastric, head and neck, hepatocellular, lung, neuroblastoma, ovarian, pancreatic, prostate, renal and other cancers have been keenly investigated since the 1980's. While the receptors and many downstream signaling molecules have been identified and characterized, there is still much to learn about this pathway and how its deregulation can lead to cancer and how it may be differentially regulated in various cell types. Multiple inhibitors to EGFR family members have been developed and many are in clinical use. Current research often focuses on their roles and other associated pathways in cancer stem cells (CSCs), identifying sites where therapeutic resistance may develop and the mechanisms by which microRNAs (miRs) and other RNAs regulate this pathway. This review will focus on recent advances in these fields with a specific focus on breast cancer and breast CSCs. Relatively novel areas of investigation, such as treatments for other diseases (*e.g.*, diabetes, metabolism, and intestinal parasites), have provided new information about therapeutic resistance and CSCs.

Keywords: EGFR, HER2, mIRs, Cancer Stem Cells, Drug Resistance, Metastasis.

EPIDERMAL GROWTH FACTOR RECEPTORS AND THEIR TARGETS IN CANCER.

The epidermal growth factor receptor (EGFR) family of receptors plays prominent roles in normal cellular growth as well as malignant transformation, prevention of apoptosis, drug resistance, cancer stem cells (CSC) and metastasis in many types of cancer [1]. The EGFR family consists of four members of membrane-spanning growth factor receptors (EGFR1, HER2, EGFR3 and EGFR4). There are multiple names for some of the family members. In this manuscript, we refer them as EGFR1 (a.k.a., EGFR, HER1, c-erbB1), EGFR2 (a.k.a., c-erbB2, HER2), EGFR3 (a.k.a., c-erbB3, HER3) and EGFR4 (a.k.a., c-erbB4, HER4). EGFR family members can induce many signaling pathways including PI3K/PTEN/Akt/mTORC1, Ras/Raf/MEK/ERK, Jak/STAT, JNK as well as others. Mutations and genetic alterations occur at diverse genes in various cancers which lead to the abnormal expression of these and other downstream pathways [2-5]. microRNAs (miRs) and long non coding RNAs (lncRNAs) regulate components of these pathways. A diagram of the EGF family of receptors and how they can heterodimerize with each other is presented in Fig. (**1**).

Various inhibitors of the EGFR family of receptors have been developed and many are being used in various cancer treatments [6]. A common EGFR inhibitor is the antibody against HER2, Herceptin® (trastuzumab), which has been used clinically to treat HER2+ breast and other cancer patients [7]. There are 622 clinical trials listed on ClinicalTrials.gov for Herceptin and breast cancer [8]. Herceptin has also been evaluated on other types of cancer including gastric [9] and ovarian cancer [10].

The effects of combining Herceptin and paclitaxel with carboplatin or epirubicin have been examined in a phase 2 clinical trial for clinical stage II-III, HER2-postitive breast cancer. Two regimens were established: paclitaxel+carboplatin+Herceptin (PCH) and paclitaxel+epirubicin+Herceptin (PEH). The main endpoint was the pathologic complete response (pCR) rate. No significant

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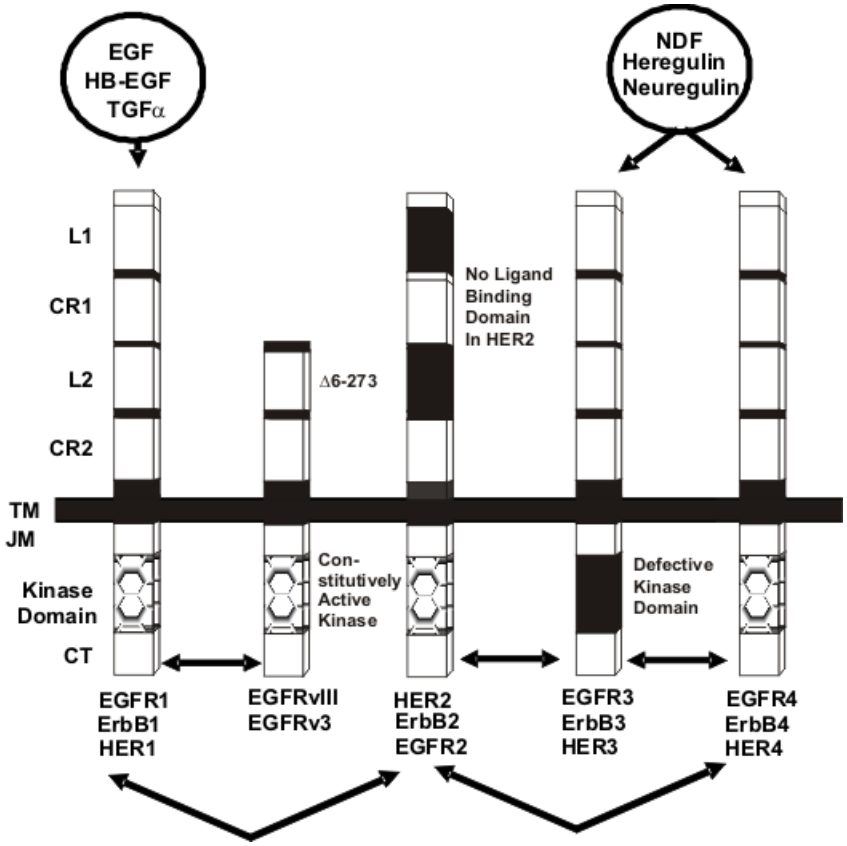

Fig. (1). Diagram of EGFR family members and various interactions between EGFR family members which may result in altered signaling transuction pathways. Key domains in these receptors are represented. Domains of the four different EGFR family members which are conserved are depicted by similar shading. L = ligand binding domain, CR = cysteine-rich domains. TM = transmembrane domain. CT = C-terminal domain which contains the phosphorylation sites. JM = juxta membrane domain. HER2 does not bind a ligand. The in frame 2-7 exon (Δ6-273) deletion in EGFRvIII is depicted. The kinase domain in the truncated EGFvIII receptor is constitutively-active. The kinase domain in ERB3 is defective. Alternative names for each receptor are written underneath each receptor. These alternative names are presented on this figure as they are still used by some authors and the reader should be familiar with them to know that multiple abbreviations can indicate the same receptor. Some of the more common growth factors and cytokines which bind the EGFRs are indicated in circles above the receptors. Epidermal growth factor (EGF), transforming growth factor-alpha (TGFα), heparin binding-EGF (HB-EGF), Neu differentiation factor (NDF), heregulin, neuregulin. Arrows between receptors indicate possible heterodimer formation between various EGFR family receptors. The mechanisms of activation of the EGFR receptors and heterodimerization have been recently discussed [2,5,14].

difference was observed between the two regimens with regards to pCR. However a higher pCR was observed in luminal-B, HER2+ and phosphatidylinositol-4,5-bisphosphate 3-kinase (*PIK3CA*) mutant patients in the PEH regimen. PEH treatment was less likely to increase the incidence of acute cardiac events compared to PCH treatment [11].

Antibodies to HER2, EGFR1, EGFR3 have been developed. These include pertuzumab, cetuximab, panitumumab, zalutumumab, nimotuzumab, necitumumab, RO5083945, MEHD7945A. Fig. (**2**) presents a diagram of antibodies targeting these receptors (EGFR1, HER2, HER2/EGFR, EGFR1/EGFR3) as well as small molecule membrane permeable inhibitors targeting the kinase domains.

The antibody pertuzumab inhibits HER2 homo-dimerization as well as HER2/EGFR3 hetero-dimerization [12-14]. Pertuzumab has been evaluated in breast cancer [15]. There are at least 69 clinical trials listed for pertuzumab and breast cancer on ClinicalTrials.gov [16]. Pertuzumab has also been evaluated in other cancers including gastric cancer [17].

Cetuximab (Erbitux®) is an antibody that targets EGFR1. It has been evaluated in the treatment of various cancer types, including triple negative breast cancer (TNBC), where EGFR is often overexpressed [18]. Cetuximab has been evaluated in at least 15 clinical trials with breast cancer patients, often in combination with other drugs [19]. Cetuximab has also been evaluated in *KRAS* wild type (WT) colo-rectal cancer (CRC) [20, 21], head and neck squamous cell carcinomas (HNSCC) [22], non small cell lung cancer (NSCLC) [23, 24] and pancreatic cancer [25].

Panitumumab is an antibody that targets EGFR1. It has been evaluated in at least four clinical trials with HER2-, TNBC and metastatic breast cancer [26]. It has been tested against other types of human cancers including CRC [27-29], gastric cancer [30] and HNSCC [31].

Zalutumumab inhibits EGFR1 signaling and has been evaluated in HNSCC [32]. Nimotuzumab is a monoclonal antibody that inhibits EGFR1 [33]. Nimotuzumab in combination with docetaxel and capecitabine is being evaluated for efficacy against breast cancer in a clinical trial [34]. It also is being evaluated in various cancers including esophageal cancer [35], glioma [36-38], gastric cancer [40], HNSCC [41] NSCLC [42], oral carcinoma [43] pancreatic cancer [44] and prostate cancer [45].

Necitumumab is also an antibody that targets EGFR1 [46]. There is one clinical trial evaluating the therapeutic efficacy of necitumumab and gemcitabine-cisplatin in advanced solid cancer patients [47]. The anti tumor efficacy of Necitumumab also has

been examined in NSCLC [48]. RO5083945 is an anti-EGFR1 MoAb. It is being evaluated in HNSCC [49].

MEHD7945A is an anti-HER3/EGFR MoAb with dual binding specificity that targets both EGFR3 and EGFR1 [50]. MEHD7945A has been evaluated in TNBC [51]. MEHD7945A was determined to suppress acquired resistance to EGFR inhibitors and radiation in HNSCC [52].

A take home message at this point is that certain members of the EGFR family have been targeted by different antibodies in various cancers. Some of the antibodies are clinically used to treat various cancers, while some are being further evaluated in clinical trials. Further development of antibodies to target EGFR family members may increase therapeutic effectiveness as in some cases combination of two antibodies targeting the same EGFR family member have proven beneficial.

There are also small molecule membrane permeable kinase inhibitors that suppress the kinase activity of certain EGFR family members and key downstream pathways. Figure **2** depicts that some of these small molecule inhibitors inhibit these kinases in these signaling pathways: Erlotinib (Tarceva®), gefitinib (Iressa®), icotinib, dacomitinib (PF-00299804), the dual EGFR1 and HER2 inhibitors, lapatinib (Tykerb®) and afatinib (Gilotrif®), the multi-targeted EGFR1, HER2 and histone deacetylase inhibitor CUDC-101, and the EGFR1, vascular endothelial growth factor receptor (VEGFR) and the rearranged during transfection (RET) oncogene-tyrosine kinase inhibitor vandetanib (CAPRELSA®). TAK-285 is a new orally bioavailable EGFR1/HER2 kinase inhibitor which penetrates the blood brain barrier. TAK-285 treatment of cells results in reduced levels of phospho-EGFR3. Phosphorylated-EGFR3 was determined to be responsible for sensitivity to TAK-285 [53].

Erlotinib has been examined in at least 29 clinical trials with breast cancer patients [54]. Erlotinib has also been evaluated in various cancers including CRC [55], hepatocellular carcinoma (HCC) [56], HNSCC and oral cancers [57], NSCLC [58], pancreatic cancer [59, 60] ovarian cancer [61].

Icotinib is a specific EGFR1 inhibitor [62]. There is at least one clinical trial with TNBC and Icotinib [63]. It has also been evaluated in NSCLC patients [64, 65]. Interestingly it was found that Icotinib interfered with ATP-binding cassette sub-family G member 2 (ABCG2)-mediated multidrug resistance in NSCLC [66].

There are at least 28 clinical trials with gefitinib and breast cancer [67]. Gefitinib has also been evaluated in the treatment of other types of cancer including glioma [68, 69], HNSCC [70] and NSCLC [71-73].

Afatinib (Gilotrif®) is an irreversible covalent inhibitor of EGFR1 and HER2. Afatinib has been evaluated in cancers including breast [74] and NSCLC [75, 76]. There are at least sixteen clinical trials with Afatinib and breast cancer patients [77].

Lapatinib is a dual EGFR1 and HER2 inhibitor which has been evaluated in breast cancer patients [78-80]. There are at least 207 clinical trials listed for examining the effects of lapatinib in breast cancer patients [81]. It has also been evaluated in various cancers including biliary tract cancer [82], cervical cancer [83], chordomas [84], esophageal cancer [85, 86], gastric cancer [87, 88], glioblastoma [89, 90], HCC [91], HNSCC [92, 93], renal cell carcinoma [94] and pancreatic cancer [95].

Recently the effects of lapatinib and all-trans retinoic acid (ATRA) on miR networks in SKBR3 breast cancer cells have been described. SKBR3 has HER2/RARA co-amplification. These cells are sensitive to ATRA and lapatinib. Such information may be im-

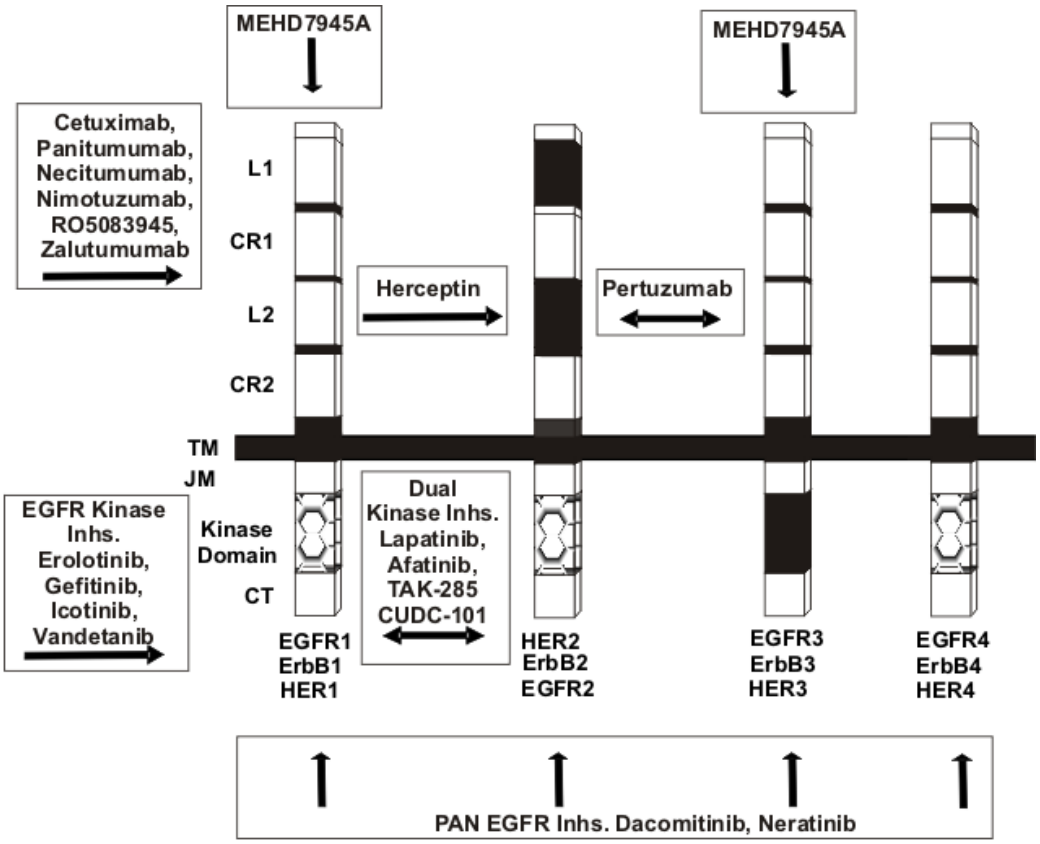

Fig. (2). Antibodies and small molecular inhibitors targeted to EGFR family members. Shown in boxes are antibodies and small molecular inhibitors which target various EGFR family members. Some of these antibodies and small molecular inhibitors target more than one family member.

portant in determining which types of breast cancer will be sensitive to lapatinib in combination with other drugs [96].

Dacomitinib (PF-00299804) is a pan EGFR inhibitor [97]. It is being examined on various cancers, such as bladder cancer [98], breast cancer [99], gastric cancer [100], glioblastoma [101], HNSCC [102] and NSCLC [103].

Neratinib is an irreversible pan EGFR inhibitor. There are at least 22 clinical trials examining the therapeutic effects of neratinib in breast cancer patients [104]. Neratinib was determined to be more active in *HER2*-amplified than non-*HER2*-amplified breast cancer. Neratinib decreased activation of all four EGFR family members as well as their downstream signaling pathways. Improved effects were observed with a combined neratinib and Herceptin therapy This combination could overcome Herceptin-resistance in HER2+, estrogen receptor (ER) alpha- SKBR3 and HER2+, ERalpha+ BT474 breast cancer cells. Neratinib also decreased HER2 and phosphorylated EGFR3 in Herceptin-resistant cells. Loss of pHER2 and HER2 expression was correlated with sensitivity to neratinib [105].

CUDC-101 is a multi-targeted EGFR1, HER2 and histone deacetylase inhibitor [106]. There was one clinical trail examining the effects of CUDC-101 in breast and other types of cancer patients [107]. The effectiveness of CUDC-101 has been examined in breast, CRC, HCC, HNSCC, glioblastoma, leukemia, NSCLC, pancreatic and other cell lines [108].

Vandetanib is an EGFR1, VEGFR and RET-tyrosine kinase inhibitor. There are at least 11 clinical trials with vandetanib and breast cancer [109-111]. It also has undergone clinical trials with epithelial ovarian, fallopian tube or primary peritoneal carcinomas [112], glioblastoma [113], HNSCC [114], NSCLC [115], prostate cancers [116] and thyroid cancers [117, 118].

Some of the mechanisms of therapeutic resistance in breast cancer may arise from the functional and structural redundancy of EGFR, HER2 and EGFR3. This family of receptors contains conserved extracellular structures which are sometimes stabilized by disulfide bonds. These disulfide bonds can be therapeutic targets. Disulfide bond disrupting agents (DDAs) have been developed to target the disulfide bonds present in EGFR family members [119]. These agents may be combined with more conventional EGFR family inhibitors to enhance their effectiveness.

The order of drug treatment may be important in certain therapeutic approaches. It has been determined that addition of the EGFR inhibitor erlotinib or the EGFR/HER2 inhibitor lapatinib enhances the cytotoxic effects of DNA-damaging drugs if erlotinib and lapatinib were added prior to the DNA-damaging agents to the MCF-7 and MDA-MB-468 cell lines. This sequential addition of the EGFR/HER2 inhibitors was determined to increase caspase-8 activation by promoting pro-caspase-8 homodimerization and autocatalytical cleavage. In contrast, the co-administration of the EGFR/HER2 inhibitors and chemotherapeutic drugs did not. The EGFR/HER2 inhibitors inhibited the activation of downstream ERK while doxorubicin activated it. These studies demonstrated that ERK can normally inhibit the formation of pro-caspase-8 homodimers by phosphorylating pro-caspase-8 at S387. Thus to improve the effectiveness of combining targeted therapy and chemotherapy, it may be necessary to first determine whether the order of addition is important and second to determine the critical targets of both targeted- and chemotherapy [120].

A take home message is that there are numerous small molecule membrane soluble kinase inhibitors which have been developed to target EGFR family members. Some of these molecules may inhibit a single EGFR family member while others may inhibit two (dual inhibitor) or more (pan inhibitor) EGFR family members. Many of the EGFR family member inhibitors have been or are being evaluated in clinical trials and some are used to treat breast cancer patients and patients with other types of cancer. In some cases the effects of combination of the EGFR kinase inhibitors with chemotherapeutic drugs are being determined. Significant clinical advances in the past decade have resulted from the development of antibodies and kinase inhibitors that specifically target various EGFR family members.. The targeting of the EGFR family members in various cancer types has been extensively discussed [2,5-7,9-15,17,18,20-25,27-33,35-62,64-103,105,106,108,110-121].

BREAST CANCER AND DEREGULATION OF EGFR SIGNALING PATHWAYS DUE TO GENETIC AND NON-GENETIC ALTERATIONS.

Certain EGFRs can interact with each other as well as other receptors which are linked to breast cancer such as the neurotensin associated receptor (NTSR1). These interactions between EGFR family receptors and other receptors may alter the growth of the breast cancer as well as the sensitivity to certain small molecule inhibitors and other drugs, such as the type II diabetes drug metformin [121].

Tyrosine dephosphorylation of the EGFR has been shown to increase its potential as a therapeutic target by suppressing its interaction with the ER. The protein tyrosine phosphatase HI (PTPH1) was reported to dephosphorylate the kinase domain of EGFR. This prevented the interaction of EGFR with nuclear ER and increased the sensitivity of the breast cancer cells to tyrosine kinase inhibitors (TKIs). PTPH1 can stabilize EGFR and stimulated the accumulation of EGFR at the membrane. PTPH1 also increased the effectiveness of combination therapy of TKIs with anti-estrogens [122].

The EGFR variant III (*EGFRvIII*) is a mutant of *EGFR1* gene [123-126]. The *EGFRvIII* gene lacks exons 2 to 7 and is an in frame deletion. The truncated *EGFR1* gene has been proposed to be involved in the transformation of many types of cancers, (*e.g*., breast, brain, ovarian, prostate and others) [124-136].

Introduction of the *EGFRvIII* gene into breast cancer cells resulted in HER2 phosphorylation. This was thought to occur through heterodimerization and cross-talk [124]. MCF-7 is a widely-used ER+, TP53 functional, *PIK3CA* mutant, and caspase 3 (*CASP3*) mutant luminal, epithelial breast cancer cell line. In athymic nude xenograft experiments MCF-7/EGFRvIII cells were more tumorigenic than parental MCF-7 cells.

The EGFRvIII protein has been detected in human cancers, but apparently not in normal tissues. EGFRvIII mRNAs were seen in primary invasive breast cancer upon laser capture microdissection (LCM)/RT-PCR. EGFRvIII mRNA transcripts were observed in 67.8% of pure breast cancer cells [125]. The LCM technique can result in the recovery of "pure" cells populations by inducing the adherence of selected cells, via a laser pulse, to a thin thermoplastic film which can then be used for molecular analysis. Samples from 28 breast cancer patients were examined in this study by the LCM-RT-PCR technique. Both EGFR1 WT and EGFRvIII mRNAs were expressed in 57.1% of the infiltrating breast carcinomas. EGFRvIII mRNAs were not detected in normal breast tissues. Immunohistochemical analysis confirmed these results. Co-expression of EGFRvIII and EGFR WT proteins was observed in some human invasive breast cancer tissues but was not detected in normal breast samples.

In a subsequent study by a different group consisting of 55 breast cancer cell lines and 170 primary breast cancers, similar results were not observed. The authors concluded that expression of EGFRvIII is extremely rare in breast cancer [126].

The expression of EGFRvIII mRNA in women with breast cancer has been examined by an RT-nested PCR. EGFRvIII mRNAs were seen in peripheral blood from 30% of 33 low risk, early stage patients. EGFRvIII mRNAs were detected in 56% of 18 patients selected for neoadjuvant chemotherapy. EGFRvIII mRNAs were observed in 63.6% of 11 patients with disseminated disease but not in any of 40 control women [127]. In summary, subsequent

studies did observe EGFRvIII expression in breast cancer patients. EGFRvIII expression was detected more frequently in breast cancer patients selected for either neoadjuvant chemotherapy or breast cancer patients with disseminated disease.

EGFRvIII expression was associated with ER- or HER2+ expression in early stage patients. The expression of EGFR1 and its phosphorylation status and the presence of EGFRvIII were examined by immunohistochemistry in a study with 225 breast cancer patients. Patient outcomes were also followed [128]. 48% of the patients expressed EGFR1 and over half (54%) of the patients expressed activated (phosphorylated)-EGFR. 4% of the patients were positive for EGFRvIII. EGFR1 expression was correlated with negative hormone receptor (estrogen receptor/progesterone receptor) status, worse relapse-free survival and overall survival than patients that did not express EGFR1 [128]. The correlation between higher EGFR1 expression and lower overall survival indicates that high EGFR1 expression is a prognostic indication of an adverse prognosis. In general, p-EGFR1 and EGFRvIII are also associated with worse prognosis, however many breast cancers do not appear to express EGFRvIII. This study concluded that EGFR1 expression is an important prognostic indicator in the HER2+ and the ER-/progesterone receptor negative (PR-) subgroups. The multiple studies have resulted in different observations regarding the expression of EGFRvIII in breast cancer patients. These differences could have been due to different methods (techniques) and reagents (primers for RNA analysis, antibodies for protein expression) used to determine EGFRvIII expression.

The EGFRvIII oncoprotein may down regulate PR expression in luminal B breast tumors [129]. These tumors are ER+ but have a 4-hydroxyl tamoxifen (4HT) resistant, aggressive behavior. This subset of breast cancers frequently displays elevated EGFR1 and HER2 expression and downstream PI3K/PTEN/Akt/mTORC1 pathway activation [129, 130].

HER2 can interact with EGFRvIII [131] as well as chemokine receptor 4 (CXCR4) [132, 133]. These interactions activate signaling pathways important in migration, invasion and tumorigenesis. It is more difficult to down regulate the constitutive, ligand-independent nature of EGFRvIII. Thus the signaling complexes induced between EGFRvIII and HER2 and CXCR4 may be prolonged in comparison to interactions between EGFR1 and HER2 and CXCR4. It turns out that CXCR4 is highly expressed in breast cancers, implicated in metastasis, CSCs and resistance to targeted therapy such as lapatinib [134, 135].

EGFRvIII expression is associated with the Wnt/beta-catenin pathway and downstream target gene expression. The expression of these genes is associated with self-renewal. EGFRvIII expression has been linked with CSC phenotypes and increased *in vitro* mammosphere formation and tumor formation [136].

HER2 is overexpressed in approximately 15-30% of women with breast cancer [2,5-7]. Overexpression can be due to gene amplification or increased transcription of the *HER2* gene. A problem with Herceptin therapy is the development of resistance. The disintegrin and metalloproteinase domain-containing protein-10 (ADAM-10) and ADAM-12 have been shown to be responsible for Herceptin-resistance in certain breast cancers. ADAM-10 and ADAM-12 play roles in maintaining HER2 phosphorylation. Herceptin treatment increased ADAM-10 levels in HER2+ breast cells which correlated with decreases in Akt phosphorylation. Knockdown of ADAM-10 enhanced the responses of HER2 sensitive and resistant breast cancer cells to Herceptin. Higher levels of ADAM-10 were associated with a poorer relapse-free survival in some breast cancer patients [137].

The CDK inhibitor p57 ($p57^{Kip2}$) is a downstream target of Akt and is important in HER2-mediated tumorigenicity. Akt is a negative regulator of $p57^{Kip2}$ as elevated Akt expression decreases $p57^{Kip2}$ expression while inhibition of Akt results in $p57^{Kip2}$ stabilization. Akt phosphorylates $p57^{Kip2}$ on S282 and T310 and Akt promotes cytoplasmic localization of $p57^{Kip2}$. HER2/Akt activation results in increased turnover of $p57^{Kip2}$ by ubiquitination. This results in HER2-mediated cell proliferation. In contrast, HER2+ breast cancer patients with high levels of $p57^{Kip2}$ had better overall survival [138].

The pre-mRNA splicing factor 4 kinase PRP4K (PRPF4B) is a component of the U5 small nuclear ribonucleic proteins (snRNP) which combine with pre-mRNAs and form the spliceosome involved in mRNA splicing. This complex can regulate the spindle assembly checkpoint (SAC), which is important in the response of cells to microtubule-targeting drugs such as taxanes, a compound used to treat breast and other cancer patients. A positive association between PRP4K and HER2 status was observed in breast tumors. HER2 signaling was determined to regulate PRP4K. Suppression of PRP4K expression was observed to reduce the sensitivity of the breast cancer cells to taxanes. Low expression of PRP4K was associated with taxane resistance in both *in vitro* studies and breast cancer patients [139]. The association between PRP4K and HER2 also was determined at the expression level in breast cancer patient samples. In addition, experiments were performed with breast cancer cell lines to determine the effects of PRP4K silencing. Knock down of PRP4K reduced the sensitivity of breast cancer cell lines to taxanes.

Recently novel targets have been identified by lentiviral shRNA kinome screening of genes involved in HER2+ breast cancer in nonadherent HER2 tumorspheres. The tumor necrosis factor receptor-associated factor (TRAF) family member-associated NF-kappa-B activator (TANK)-binding kinase 1 (TBK1) gene is a non-cannonical I-kappa-B kinase. The TBK1-II drug inhibited TBK1 and it suppressed the proliferation and induced senescence of HER2+ cells. These studies determined that the TBK1 inhibitor cooperated with the dual inhibitor lapatinib in inhibiting growth in a xenograft model. Thus this combined therapeutic approach may be appropriate for certain cancers [140].

The expression and roles of the EGFR3 receptor in breast and other cancers are complex. EGFR3 lacks a kinase domain. EGFR3 binds the ligands heregulin and neuregulin 2 (NRG-2). Binding of these ligands results in the possibility of dimerization with another EGFR family member and a conformation change which results in activation of downstream signaling. EGFR3 can heterodimerize with any of the other three EGFR family members [141]. See Fig. (**1**).

Treatment of luminal breast cancers with the antiestrogen fulvestrant (FASLODEX®) results in EGFR3 upregulation. EGFR3 can then result in activation of the PI3K/PTEN/Akt/mTORC1 pathway and enhanced survival of breast cancer cells [142]. The expression of the leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) protein, a transmembrane protein which interacts with EGFR family members and other receptor tyrosine kinases, correlated positively with ERalpha, but inversely with EGFR3. LRIG1 is an estrogen-inducible EGFR down regulator. Overexpression of LRIG1 resulted in enhanced fulvestrant-mediated growth inhibition. In these studies, LRIG1 expression correlated positivity with disease-free survival in antiestrogen treated patients. LRIG1 expression suppressed EGFR3 signaling in luminal breast cancers. In contrast, if ERalpha signaling was suppressed, LRIG1 signaling was repressed which resulted in enhanced EGFR3 activity [142].

The role of EGFR4 in breast cancer remains complex [143, 144]. EGFR4 expression is associated with improved outcomes in certain breast cancer patients undergoing certain therapies. HER2+ patients who also expressed EGFR4 exhibited a significant delay in the development of metastasis after Herceptin treatment [145]. Furthermore these patients exhibited a significant improvement in progression free survival (PFS) after Herceptin treatment.

A high specificity EGFR4 Ab (E200) has been developed [145]. With this antibody, it was determined that patients with HER2/EGFR4 expression displayed a delay in the development of breast metastasis after neo-adjuvant Herceptin therapy as well as an improvement in PFS.

Recent studies have indicated that nuclear EGFR4 is associated with resistance to Herceptin and linked with a poor outcome in HER2+ breast cancer. It is possible to prevent EGFR4 cleavage with a secretase inhibitor and inhibit EGFR tyrosine kinase activity by neratinib treatment. This combination treatment resulted in decreased EGFR4 nuclear translocation and enhanced the response to Herceptin. Thus preventing the nuclear translocation of EGFR4 is important in Herceptin sensitivity [146].

Recently it has been shown by analysis of 238 primary invasive breast cancer patients that the localization of the intracellular domain of EGFR4 and the presence of certain alternatively-spliced exons of EGFR4 can have prognostic significance in ER+/HER2- breast cancers [147].

A take home message for this section is that roles for all four of the EGFR family members as well as EGFvIII have been described in breast cancer. In some cases increased expression of EGFR family members is associated with certain breast cancer types (HER2, EGFR1) which can lead to specific treatment with antibodies and small molecule inhibitors that target these EGFR family members. In contrast, in some cases EGFR4 expression may be associated with a better prognosis.

BREAST CANCER STEM CELLS/CANCER INITIATING CELLS (CSCS/CICS)

The concept of a subset of cancer cells that are responsible for efficient tumor growth has emerged over the past 20 years [148]. There are multiple terms and abbreviations for CSCs and CICs. Other nomenclatures include: tumor initiating cells (TIC), stem/progenitor cells and CSC-like cells. In this review, we will use the abbreviations used by the authors in their original manuscripts. CSCs may be responsible for cancer initiation and growth after various therapeutic approaches and they have the critical capacity for self-renewal of the tumor. They can result in the generation of heterogeneous lineages of cancer cells that comprise the tumor. CSCs may arise from various sources including stem, progenitor or differentiated cells [149]. They are frequently resistant to classical cancer treatments, such as chemotherapy, radiation and surgery, and may remain after "standard" treatments. CSCs evolve, or become selected for, after targeted therapeutic approaches.

CSCs were originally characterized in acute myeloid leukemia (AML) cells, but have since been observed in numerous other cancer types including breast, cervical, colorectal, glioblastoma, HCC, medulloblastomas, melanoma, oral, pancreatic, prostate and others [148, 150-154]. CSCs are found in 0.1 to 1% of the cells in an AML. The CSCs have a cell cycle phenotype similar to stem cells [155]. CSCs differ from the bulk of the tumor cells (BC) present in the cancer patient. CSCs are usually dormant or slowly replicating. They can undergo asymmetric division to produce cells with certain stem cell properties or produce a more differentiated BC. Genomic profiling has revealed that CSCs have gene expression patterns more similar to stem cells than to the BCs [156]. Some CSCs have enhanced telomerase (TERT) and DNA repair activities as well as high levels of membrane bound ATP-binding cassette transporters (ABC "drug" transporters) whose normal functions are to exclude xenobiotics. CSCs and stem cells have been defined in some cell types and patient samples by their increased ability to exclude dyes (Hoechst 33342) and thus on FACS analysis they are referred to as the side-population [14,157]. Unfortunately CSCs may repopulate the patient with a more aggressive, metastatic or drug resistant cancer [2,5,14,151,158]. Development of effective targeting techniques that inhibit and eliminate CSCs is a critical area of research.

In a study with 448 primary breast tumors, CD44 expression was associated with high grade, ER-, PR- tumors. CD44 is the hyaluronan receptor. CD44 expression was also associated with increased distant recurrence and reduced disease-free survival in certain patients with either large or lymph-node positive tumors. It was shown that by suppressing CD44 expression with shRNAs, adherence of TNBC MDA-MB-231 cells to endothelial cells and invasion were decreased; however, cell proliferation was not [158].

The CD44+CD24-/low phenotype is one of the most well characterized phenotype of breast CSCs/CICs. This CD24 gene encodes a sialoglycoprotein, a cell adhesion molecule. The CD44+CD24-/low phenotype has been evaluated as a prognostic marker in certain breast cancer patients that were treated with chemotherapy. In a study with breast cancer samples obtained from patients that had undergone mastectomy followed by chemotherapy, it was determined that the majority of patients with CD44-/CD24- or CD44-/CD24+ were ER+ patients. Patients with tumors that were CD44+/CD24- may be sensitive to chemotherapy with taxanes and/or anthracyclines [159].

The expression of ER, PR, EGFR1, HER2, CD44, CD24, CK5, CK14 and ALDH1 was examined in 364 familial breast cancer specimens. CD44+CD24-/low was observed in 16% of the samples and associated with a high tumor grade. Likewise 15% of these breast cancers were ALDH1+ and associated with a high tumor grade. CD44+CD24-/low and ALDH1+ expression were associated with a basal-like subtype [160].

Activators of CD44 have been identified. The AF1q protein is involved in the formation of transcriptional complexes. It is encoded by a gene (*MLLT11*) originally identified in a chromosomal translocation in mixed-lineage leukemia. AF1q binds to the T-cell-factor-7 which is part of the beta-catenin transcription complex frequently involved in Wnt signaling. When AF1q and TCF-7 interact, CD44 is transcribed. This can result in breast cancer metastasis. This recent study demonstrates the importance of CD44 in breast cancer metastasis [161].

The Cripto-1 protein may be a novel therapeutic target for TNBC. Cripto-1 is an embryonic stem cell marker. Cripto-1 proteins may act as co-receptors or ligands. Knock-out of Cripto-1 by the CRISPR-Cas9 system suppressed TNBC growth and metastasis [162].

Recently it has been shown that the cleaved intracellular domain of CD44 (CD44ICD) can activate the expression of NANOG, SRY (Sex Determining Region Y)-Box 2 (Sox2) and octamer-binding transcription factor 4 (Oct4) also known as POU domain, class 5, transcription factor 1 (POU5F1). The protein products of these genes are associated with stemness. Activation of these genes has been associated with tumorigenesis. Overexpression of the CD44ICD was shown to increase mammosphere formation. Prevention of cleavage of CD44ICD by treatment with a gamma-secretase inhibitor (GSI) was shown to prevent mammosphere formation. Nuclear localization of CD44ICD was determined to be important for the transcriptional activation of the stemness genes. These studies demonstrated the importance of the CD44ICD in breast CSC and suggest a potential therapeutic target [163].

A take home message for this section is that there are breast cancer stem cells that show certain similar characteristics as other cancer stem cells and stem cells. While CD44 high and CD24 low has been associated with breast CSCs for some time now, some novel genes are being described to be important in breast CSCs.

SIGNALING PATHWAYS IN BREAST CSCS AND THERAPY RESISTANCE

Some breast CSCs are ERalpha-. They may rely on paracrine signals derived from the mammary epithelium for development. E2 was determined to stimulate CSC activity in CD44+, CD24low, epithelial-specific antigen positive (ESA+) cells which were de-

rived from ER+ breast cancer patients. The cells had low or no ER expression. However E2 was determined to stimulate mammosphere formation and tumor growth. The authors concluded that E2 mediated its effects via paracrine signaling from non-CSCs to CSCs. The EGFR1 inhibitor geftinib and a Notch gamma-secretase inhibitor suppressed E2-mediated CSC activity. These studies point to the interactions between E2, EGFR and Notch signaling in ER-CSCs [164].

Notch hyper-activation has been shown to drive mesenchymal transition in hormonal therapy resistant breast cancer via the *Drosophila melanogaster* X-like-2 (DMXL2) gene product. DMXL2 is a regulator of Notch signaling. DMXL2 is overexpressed in certain endrocrine-therapy resistant breast cancers and it promoted epithelial mesenchymal transition (EMT) via Notch. DMXL2 protein levels were determined to increase in ERalpha+ patients who progressed after endocrine therapy. These studies have suggested that DMXL2 may be a marker for ERalpha+ breast cancer patients who progress [165].

The RNA binding protein RNPC1 can post-transcriptionally regulate ERalpha. RNPC1 binds the ERalpha mRNA and stabilizes it. A regulatory loop was identified when overexpression of ERalpha decreased RNPC1 mRNA and protein levels [166].

Targeting EGFR1, HER2 and Notch was determined to be effective in suppressing ductal carcinoma *in situ* (DCIS) stem/ progenitor cell activity. These studies used DCIS cell lines MCF-10DCIS.com, [HER2 normal] and SUM225, [HER2overexpressing] and patient samples which differed in HER2 expression. Treatment with the Notch inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycinet-butyl ester) and the dual EGFR1/HER2 inhibitor lapatinib or EGFR1 inhibitor gefitinib was determined to be more effective in suppressing stem/progenitor cell activity. These studies indicate that targeting Notch and EGFR1/HER2 may be effective even in the cases of low or no HER2 expression [167].

Hypoxia inducible factor-1alpha (HIF-1alpha) and HIF-2alpha are important for CIC survival especially during oxygen and nutrient deprivation. The HIFs may induce the expression of many genes that are important in CIC survival, energy metabolism, invasion and metastasis. Many signaling pathways including: EGFR, insulin like growth factor-1 receptor (IGF-1R), stem cell factor receptor (Kit), TGF-beta and Notch as well as their downstream associated signaling components may regulate the activity of the HIFs [168].

Recently it has been determined that a subset of HER2-positive breast cancers, which expresses urokinase plasminogen activator receptor (uPAR), has been determined to have properties of stem and mesenchymal cells with regard to motility and metastasis [169].

EMT has been shown to confer resistance to the mTOR blocker rapamycin. E-cadherin is a protein associated with epithelial cells and is detected in rapamycin-sensitive cells. Mesenchymal breast cells are more resistant to rapamycin. MCF-7 cells are normally sensitive to rapamycin. However, when MCF-7 cells were transfected with constitutively-active Snail, a transcription factor important in EMT, they became rapamycin-resistant whereas if they were transfected with WT Snail, they remained rapamycin-responsive. When rapamycin-resistant mesenchymal ACHN cells (derived from a kidney disease patient) and MDA-MB-231 cells (TNBC) were transfected with miR-200b/c or ZEB1 siRNA, aspects of mesenchymal to epithelial transition occurred. Increased levels of E-cadherin were observed in both cell lines and ACHN cells became sensitive to rapamycin. Treatment of the ACHN and MDA-MBA-231 cells with the MEK inhibitor trametinib and rapamycin inhibited xenografts in mice, but did not appear to inhibit EMT in those cells [170]. In certain cell types, both the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR/GSK-3 pathways may regulate the activity of Snail. Both of these pathways are regulated by EGFR and both pathways also regulate EGFR activity [171].

Poly(ADP-ribose) polymerase-1 (PARP-1) also may be important in the regulation of Snail. PARP inhibitors may affect Snail activity. MD-MBA-231 cells are often considered resistant to chemotherapeutic drugs and have a high frequency of CD44+/CD24- cells that are often associated with a phenotype of breast CSCs. The PARP inhibitor ABT-888 was able to augment the response of MDA-MB-231 cells to doxorubicin. These results are relevant to both therapy of TNBC and breast CSCs [172].

The cellular localization of AKT affects the maintenance of breast CSCs. Introduction of a modified *AKT* gene tagged with the nuclear localization signal into SKBR3 and MDA-MB-468 TNBC cells resulted in increased number of cells with the CD44+/CD24- and ALDH+ phenotypes. These effects could be reversed upon treatment with the AKTinhibitor triciribine [173].

The novel ERK inhibitor (BL-EI001) has been shown to have anti-proliferative properties in breast cancer. BL-EI001 was shown to induce apoptosis in breast cancer cells by the mitochondrial pathway that was independent of Raf/MEK/ERK pathway activation. BL-EI001 was determined to inhibit ERK phosphorylation [174].

The MEK5/ERK5 pathway may be an important target in TNBC. It has been proposed that this pathway is a therapeutic target in breast cancer and inhibition of ERK5 and combination with chemotherapeutic drug treatment may be an effective therapeutic approach for certain breast cancers [175-177].

EGFR is frequently overexpressed in TNBCs. EGFR can induce the Raf/MEK/ERK pathway [2,5]. The ribosomal six kinase (Rsk) lies downstream of ERK. TNBCs are enriched in CSCs. A high percentage of the cells in certain TNBC cell lines display elevated CD44+/CD24- phenotype. TNBCs also have the highest rate of recurrence. Rsk has been shown to be important in the growth and proliferation of CSCs [178]. Recently a novel inhibitor to Rsk (LJI308) has been developed [179]. LJI308 was recently shown to target the CSC population and inhibit TNBC growth [180].

Insulin-like growth factor-1 receptor (IGF-1R) and focal adhesion kinase (FAK) signaling are important in metastatic breast cancer [181]. The expression of the IGF-1R has been shown to be essential for maintaining the mesenchymal properties of TNBCs. IGF-1R was determined to upregulate EMT markers as well as promote migration and invasive properties in the TNBCs. The migration and invasive properties promoted by IGF-1R were determined to be mediated by FAK activation [182].

Protein kinase C-alpha (PKC-alpha) has been shown to be important in breast CSCs. Inhibition of PKC-alpha suppressed breast CSCs but had fewer effects on non-CSCs. During the generation of CSCs from non-stem cells, it was shown that there was a shift from EGFR to platelet derived growth factor receptor (PDGFR) signaling which resulted in activation of PKC-alpha. PKC-alpha induced the Fos related antigen-1 (FRA-1) transcription factor. PKC-alpha and FRA-1 expression were determined to be associated with TNBC. Depletion of FRA-1 was shown to result in a mesenchymal-epithelial transition (MET). These studies also determined that FRA-1 was utilized in CSCs while c-FOS was important in non-CSCs [183].

A take home message for this section is that many signaling molecules play important roles in breast CSCs. The effects of these molecules on therapeutic resistance are being elucidated. There exist potent inhibitors for certain signaling molecules which may eventually be translated into therapeutic approaches.

TARGETING EGFR FAMILY MEMBERS AND ER ACTIVITY IN BREAST CANCER AND BREAST CANCER CSCS

The effectiveness of treating HER2 or HER2/low patients with anti-HER2 therapies has been re-examined recently. It was observed that HER2/low tumors might be inhibited by agents that suppress HER2. This may result from HER2/EGFR3 signaling in

the breast CSCs by the neuregulin-1 (NRG1) produced by the breast tumor initiating cells (TIC). This NRG1 is believed to suppress proliferation in HER2/low cells and TNBC. Inhibition of EGFR1 and/or HER2 increased the sensitivity of the breast TICs to radiation. These results suggested that inhibition of EGFR1, HER2 and EGFR3 might prove effective in the treatment of breast cancers that were not previously thought to be sensitive to anti-EGFR1/HER2 therapeutics [184].

The effects of inhibiting CXCR1 and CXCR2 were examined in patient derived breast cancer specimens. CXCR1 and CXCR2 are the cognate receptors for interleukin-8 (IL-8) that have been determined to be upregulated in breast cancer and associated with poor prognoses. IL-8 affected mammosphere formation in metastatic and invasive breast cancers. IL-8 activated EGFR1 and HER2 signaling pathways. Inhibitors of EGFR1, HER2, SRC, PI3K or MEK suppressed the effects of IL-8. Lapatinib inhibited IL-8 induced mammosphere formation in both HER2+ and HER2- breast cancer cells. Inhibition of CXCR1 and CXCR2 suppressed the effects of IL-8 and increased the potency of lapatinib on mammosphere formation in HER2+ breast cancer cells [185].

Mucin 1 (MUC1-C) is a cell surface mucin that has O-linked glycosylation at its extracellular domain. The MUC1-C oncoprotein is important in the self-renewal properties of breast mammospheres. MUC1-C was shown to activate NF-kappaB and induce IL-8 transcription as well as IL-8R and CXCR1 expression. Targeting MUC1-C with the cell penetrating peptide GO-203, which binds the MUC1-C cytoplasmic domain and blocks MUC1-C function, prevented IL-8/CXCR1 expression as well as mammosphere formation. Targeting MUC1-C cell is a potential therapeutic approach [186].

Recently lapatinib-resistant breast cancer cells have been derived. It was determined that Src and CXCR4 were involved in the resistance to lapatinib as well as the invasiveness of the cells [187]. CXCR4 is increased in anoikis-resistant breast cancer cells. Anoikis-resistant breast cancer cells had enhanced mammosphere formation and tumor forming ability compared to unsorted cells. CXCR4 mRNA was detected at higher levels in CD44+/CD24- cells from breast cancer patients than from non-enriched cells. Interestingly, treatment of the patient derived breast cancer cells as well as the ERalpha+ T47D breast cancer cell line with the ligand for CXCR4, SDF-1, increased mammosphere formation ability. Whereas treatment of normal breast cells with SDF-1 reduced mammosphere formation ability. These important studies indicate that CXCR4 may play important roles in breast cancer stem activity, tumor formation and metastases [188].

ER+ breast cancers are often treated with aromatase inhibitors (AIs), however resistance to therapy can develop. Resistance can result from increased dependence on HER2 signaling. Certain AI-resistant cells overexpressed both HER2 and breast cancer resistance protein (BCRP) and had increased levels of TICs compared to AI-sensitive cells. Inhibition of HER2 and BCRP decreased the TIC characteristics in AI-resistant cells. These studies also documented an interaction between EGFR1 and HER2 involved in BCRP regulation [189].

A take home message for this section is that the EGFR family members and ER activity have been associated with both therapy resistance and CSC formation in breast cancer. Additional signaling and drug resistance pathways are being shown to interact with the EGFR family members and ER pathways to influence CSC development. Often these additional pathways may induce cytokine or chemokine signaling which may confer resistance as well as the ability to form mammospheres.

MUTATION/ACTIVATION/INACTIVATION OF SIGNALING PATHWAYS MEMBERS IN BREAST CANCER

The PI3K/PTEN/Akt/mTORC1 pathway is frequently altered in breast cancer, in part due to activating mutations in *PIK3CA* and inhibition of PTEN activity by multiple mechanisms. A grouping of some oncogenes and tumor suppressor genes important in breast cancer is presented in Figure (**3**). In a study by Wheler *et al*, the mutational status of key genes in this pathway was examined in 57 women with metastatic breast cancer. 216 mutations were observed in 70 genes [190]. The most common gene alterations observed in this study included: *TP53,* PI3K (*PIK3CA*), Cyclin D (*CCND1*), avian myelocytomatosis viral oncogene homolog (*MYC*), *HER2,* myeloid leukemia cell differentiation protein (*MCL1*), phosphatase and tensin homolog (*PTEN*), fibroblast growth factor receptor 1 (*FGFR1*), GATA binding protein 3 (*GATA3*) neurofibromin 1 (*NF1*), phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (*PIK3R1*), breast cancer 2 (*BRCA2*), *EGFR,* insulin receptor substrate *2* (*IRS2*), cadherin 1, aka E-cadherin (*CDH1*), cyclin-dependent kinase inhibitor 2A (*CDKN2A* encodes p16INK4 and p14ARF), fibroblast growth factor 19 (*FGF19*), *FGF3* and *FGF4*. Many different types of genetic alterations were observed in this study which included: mutations, amplifications, deletions, splicing mutations, truncations, fusions and rearrangements. In some cases, there were multiple alterations in the same gene. This study documents the important roles of gene mutations in breast cancer and also indicates the possibility of therapeutic intervention with precision medicine as many of the genes encode proteins which are drugable targets.

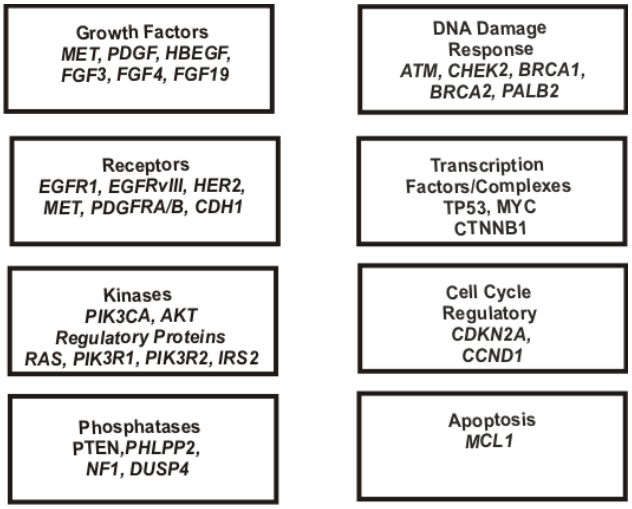

Fig. (3). Types of genes implicated in breast cancer. Mutation or aberrant expression of many different signaling pathways can result in altered signaling and contribute to cancer progression. Key genes implicated in breast cancer are classified into groups. Additional genes are implicated in breast cancer.

Patients who have *BCRA1* mutations have a predisposition to basal-like breast cancers. These patients have a poor prognosis due to tumors that have a high metastatic rate. The *BRCA1* gene product has been shown to suppress EMT; disruption of the *BRCA1* gene induced EMT in *p18(Ink4c)* mutant mice. This results in the de-differentiation of luminal stem cells and the expansion of basal cells and CSCs. BRCA1 can bind the TWIST promoter and suppress its activity along with EMT. In human tumors, BRCA1 was shown to be inversely correlated with TWIST expression and aspects of EMT. These studies point to the critical role BRCA1 has on TWIST expression and EMT [191]. BRCA1 expression has also been shown to be inversely correlated with ALDH1 expression. Suppression of BRCA1 expression resulted in an increase in breast epithelial cells expression of ALDH1 [192].

The partner and localizer of BRCA2 (*PALP2*), also known as *FANCN,* is another important tumor suppressor gene in breast and

other cancer types. *PALP2* encodes a gene product involved in double strand DNA repair [193, 194]. The checkpoint kinase 2 (CHEK2), encoding the serine threonine kinase 2, is also a tumor suppressor gene that plays important roles in breast cancer [195].

Next generation sequencing and microarray gene expression studies were performed on CD44+/CD24- breast CSC isolated from ER+ breast cancer patient cells. Genes involved in the EGFR1/PI3K pathway, including *EGFR1*, heparin binding epidermal growth factor (HB-EGF), *PDGFRA/B*, *PDGF*, *MET*, *PIK3CA*, *PIK3RA*, and phosphatidylinositol 3-kinase regulatory subunit beta (*PIK3R2*), were determined to be expressed at elevated levels. In addition, genes involved in the maintenance of stemness were also expressed at high levels [196].

Mutations in downstream components of the EGFR pathway can contribute to breast cancer in many cell types. Examples include loss of functional *PTEN* and activating mutations at *PIK3CA*. These mutations may confer sensitivity to drugs that target mTORC1, such as rapamycin, rapalogs, PI3K/mTOR dual inhibitors and metformin [197, 198].

In a study with 104 formalin-fixed paraffin-embedded TNBC patient samples, the mutational status of 44 key genes in the PI3K/PTEN/Akt/mTORC1, Raf/MEK/ERK and TP53 pathways was evaluated. *TP53* was mutated in more than 80% of the TNBC patient samples. *PIK3CA* mutations were detected in 29.8% of the patient samples. Amplification or deletion of PI3K-associated genes was detected in 7.7% of the patient samples. Mutations at the Raf/MEK/ERK pathway members were detected in 8.7% of the TNBC patient samples. Finally mutations in cell cycle regulators were detected in 14.4% of the TNBC patient samples examined in this group [199].

Ras-related protein Rab-1B (RAB1B) is a member of the RAS oncogene family. RAB1B was determined to be down-regulated in certain highly metastatic TNBC cells selected from the original TNBC line. Downregulation of RAB1B was found to stimulate proliferation of TNBC, *in vitro* and *in vivo*. A link between RAB1B and transforming growth factor beta receptor-1 (TGF-betaR1) was determined, as loss of RAB1B led to increased TGF-beta1R expression and SMAD phosphorylation. Low levels of RAB1B were associated with poor prognosis in breast cancer patients. RAB1B may function as a metastasis suppressor in TNBC [200].

Increased expression of the Ras GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) protein has been shown to induce EMT in breast cancer cells. The involvement of SMAD in G3BP1-induced EMT was determined. Silencing SMAD suppressed G3BP1-induced EMT. G3BP1 was determined to interact with the SMAD complex and may regulate their phosphorylation. Knock-down of G3BP1 suppressed the mesenchymal characteristics of the MD-MA-231 TNBC line [201].

Downstream in the Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTORC pathways lies proteins, such as $p21^{Cip-1}$ and $p27^{Kip-1}$, that regulate cell cycle progression and multiple transcription factors, which control gene expression by both positive and negative mechanisms. The transcriptional enhancer factor TEF-3 (TEAD4) binds Kruppel-like factor 5 (KLF5). Knockdown of either TEAD4 or KLF5 in TNBCs induced the expression of the CDK inhibitor $p27^{Kip-1}$ in TNBC cell lines. Thus in TNBC cell lines, TEAD4 and KLF5 promote proliferation at least in part by inhibiting $p27^{Kip-1}$ expression [202].

The effects of the MEK inhibitor selumetinib on MDA-MB-231 TNBC cells have been examined. MDA-MBA-231 cells have mutant *BRAF* and *KRAS* genes [203]. The MEK inhibitor selumetinib decreased the presence of MDA-MB-231 cells in xenograft studies which was associated with cavitation of the bone metastases due to apoptosis. The authors hypothesized that inhibition of MEK in certain breast cancers with Ras/Raf/MEK/ERK pathway activated may be useful in initiating cell death.

The Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways often regulate the activity and expression of anti-apoptotic pathways. Various anti-apoptotic molecules are aberrantly expressed in breast cancer, *e.g.*, MCL-1 in TNBC. The anti-apoptotic survivin molecule is a target in cancer therapy. YM155 is a small molecule inhibitor that may suppress survivin. YM155 has recently been shown to suppress primary breast cancer cells in an ex vivo assay which preserves certain aspects of the tumor microenvironment. YM155 also would induce cell death in some breast cancer cells which have stem like properties but not in non-tumorigenic mammary cells. The cell death induced by YM155 was determined to be dependent on autophagy and NF-kappaB but not TP53 [204].

Recently the involvement of specific PI3K isoforms in certain biochemical processes has gained importance [205-217]. The development of novel compounds to target specific PI3K isoforms and downstream PTEN is an emerging avenue of cancer therapy as well as other diseases [205-218].

The Kruppel-like factor 9 (KLF9) has been determined to be a suppressor of invasive growth in TNBC by microarray analysis. Overexpression of EGFP-KLF9 in MDA-MB-231 TNBC cells blocked invasion and altered morphology. Importantly KLF9 expression was determined to be inversely correlated with mitotic activity in clinical samples. These results indicate that the KLF9 may be a suppressor of invasive growth in certain breast cancer [209]. The EGFR is transcriptionally repressed in breast cancer by KLF10. There are numerous KLFs (>17) and they have been shown to play key roles in many cancers [210].

AKT and mTOR are also implicated as key signaling molecules in many cancer types including breast. In some cases, leukemia inducible factor (LIF) can induce AKT and mTOR and promote tumorigenesis and metastasis. Overexpression of LIF was associated with a poorer relapse free survival (RFS) and LIF expression could be an important prognostic marker for breast cancer [211].

The ER and PR play key roles in breast cancer and also serve as important targets and prognostic indicators [212]. ERalpha can regulate the FoxO3a transcription factor that is important for breast cancer motility and invasion. Some studies have shown opposite roles for FoxO3a as overexpression of FoxO3a decreases motility, invasiveness and anchorage-independent growth of ERalpha+ cells but has the opposite effects in ERalpha- cells. These results imply that ERalpha is a crucial switch in the ability of FoxO3a to control breast cancer aggressiveness [213].

The ER plays important roles in breast cancer by itself as well as by interacting with other signaling pathways. Long-term estrogen deprivation (LTED) is a therapeutic approach to treat breast cancer. However LTED can result in resistance to the ER down-regulator fulvestrant as the cells may exhibit activation of other kinases and growth factor receptor pathways such as IGF-1R. Combining fulvestrant with the multi-kinase inhibitor dasatinib (Sprycel®) was shown to have promising results in suppressing the growth of certain LTED breast cancer cells [214].

Adiponectin is an important protein in breast cancer and may interact with ERalpha and other important signaling molecules, such as IGF-1R and c-Src. Adiponectin is normally secreted by adipose tissue and induces insulin-sensitizing and other important properties. Recently, it was shown that adiponectin induces different effects in ER+ and ER- breast cancers as it stimulated the proliferation of ERalpha+ MCF-7 cells but suppressed the proliferation of ERalpha- MDA-MD-231 cells. Adiponectin induced MAPK phosphorylation in ERalpha+ but not ERalpha- cells and this induction was controlled by ERalpha expression. The induction of MAPK was determined to be due to IGF-1R activation by adiponectin. Adiponectin was able to transactivate the ERalpha in MCF-7 breast cancer cells which resulted in nuclear localization, downregulation of mRNA and protein levels as well as upregulation of estrogen-responsive cells [215].

Recently the BCL9-2 protein has been shown to exert effects on ERalpha expression during breast cancer tumorigenesis. It was determined that tamoxifen-treated patients with high BCL9-2 expression had better survival. BCL9-2 expression was determined to regulate ER transcription. [216].

Other signaling pathways important in breast cancer, such as prolactin (PRL) and the PRL-receptor (PRLR), also regulate ERalpha. PRLF can be activated by STAT5 binding a gamma interferon activation site (GAS)-element in the PRLR gene. ERalpha can stimulate PRLR expression. PRLR can stimulate the phosphorylation of ERalpha by the HER2/PI3K, Raf/MEK/ERK and Jak pathways [217].

The proliferation of ER+ breast cancers is dependent on mixed lineages kinases (MLK). The pan-MLK CEP-1347 inhibitor blocked cell cycle progression in G2 and early M phase of the cell cycle in three ER+ breast cancer cell lines as well as one which had acquired anti-estrogen resistance. Interesting CEP-1347 did not have similar effects on cell cycle progression in two non-tumorigenic mammary epithelial cell lines. While CEP-1347 decreased JNK and NF-kappaB activity, it did not affect the levels of active ERK or p38. These interesting studies suggest that MLK inhibitors may eventually be useful in the treatment of certain ER+ breast cancers [218].

A take home message for this section is that numerous genes have been identified to have roles in breast cancer. The involvement of some of these genes (*HER2*, *BRCA1*, *BRCA2*, *TP53*, etc) has been known for quite some time. New methods of gene analysis indicate that many additional genes and proteins are involved in breast cancer. Often the genes implicated belong to signaling pathways involved in the regulation of growth in many different types of cells, both normal and cancer cells. However, recent studies have indicated that there are some genes which are mutated more frequently in hormonally-responsive cells and tumors. Novel inhibitors have been developed to target some of the genes shown to be aberrantly expressed in breast cancer.

CONTROL OF GENE EXPRESSION BY REGULATORY RNAS IN BREAST CANCER

Micro RNAs (miRs) and long non coding RNAs (lncRNAs) have been shown to play critical roles in breast cancer and other types of cancer and may be novel, useful biomarkers for various therapeutic approaches. By analyzing gene expression data from 1488 human primary breast cancer tumors in public data sets, the zinc finger E-box-binding homeobox 1 (ZEB1) transcription factor was associated with the lysophosphatidic acid (LPA) G protein coupled receptor (LPAR1) encoding LPA1. This association was strongest in basal primary carcinomas. ZEB1 expression was determined to be regulated by the LPA1/PI3K pathway and LPA1/PI3K/ZEB1 regulated miR-21 expression. Suppression of miR-21, LPA1 or ZEB1 inhibited LPA-induced migration. High LPAR1 expression was determined to be associated with poor metastasis-free survival in basal tumors [219]. The tumor suppressive miR-200 family was not expressed in TNBC cell lines. miR-200b-3p overexpression suppressed EMT, decreased migration and increased E-cadherin expression [220]. The ZEB1 transcription factor plays important roles in the aberrant activation of EMT. Abnormal activation of EMT can contribute to invasion and metastasis. Genes that are induced by ZEB1 and expressed during breast cancer bone metastasis have been identified. ZEB1 was shown to induce the expression of the bone morphogenetic protein (BMP)-inhibitors, noggin (NOG), follistatin (FST) and chordin-like 1 (CHRDL1) by two distinct mechanisms, namely it induced their transcription and also suppressed the expression of miR-200 family members which would normally inhibit their expression. By increasing BMP-inhibitor expression and suppressing BMP signaling, ZEB1 promotes osteolysis, bone metastasis and disease [221]. The process of EMT is important in breast cancer and breast CSCs. ZEB1 can promote EMT in carcinoma cells and induce invasion and metastasis. Recently is has been proposed that ZEB1 can influence radioresistance in breast cancer cells independently of EMT [222].

The Hippo pathway is a key regulator of tissue growth and cell fate. Deregulation of the Hippo pathway has been observed in various cancers. G protein coupled receptor (GPCR) regulates the Hippo pathway via Rho GTPases [223]. Recently the tafazzin (TAZ) transcriptional co-activator was determined to be a downstream effector of the Hippo pathway. TAZ is believed to be a key driver in the malignant phenotype of TNBC. Constitutive expression of TAZ in mammary basal epithelial cells conferred the CSC phenotype. Targeting TAZ in mammary tumors may be an approach to reduce the CSC population [224].

miR-125a has been recently shown to be important in breast CSC. miR-125a targets the leukemia factor receptor (LIFR) that regulates the Hippo pathway. miR-125a indirectly targets TAZ which is an Hippo effector molecule. The miR-125-LIFR interaction was determined to be important in both non-malignant breast epithelial stem cells and malignant breast CSCs. Suppression of miR-125a led to a reduction in the breast CSC pool while enhanced expression of miR-125a increased the stem cell pool in the non-malignant breast epithelial stem cells. The localization and activity of TAZ were determined to be regulated by miR-125a [225].

miRs can act as either tumor suppressor or tumor promoters. They also have critical roles in regulation of "normal" gene expression [226-228]. A diagram depicting the effects of miRs on ER signaling is presented in Fig. (**4**). miR-181a/b can act as a negative regulator of the DNA damage response (DDR) and effect the expression of the stress-sensor kinase ataxia telagieatasia mutated (ATM). miR-181a and miR-181b were determined to be overexpressed in aggressive breast cancer. In contrast, ATM levels were lower in those breast cancers that expressed high levels of miR-181a and miR-181b [229]. A diagram depicting the effects of drugs on miRs expression is presented in Fig. (**5**).

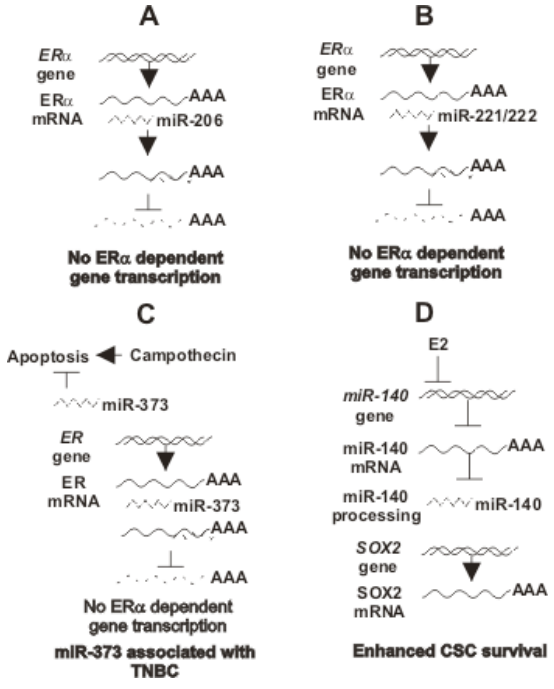

Fig. (4). Effects of MIRS on ER and E2-mediated gene expression. Panels A & B) ERalpha gene expression is suppressed by miR-206 and miR-221/222. Panel C) Effects of campothecin and miR-373 on ER gene expression. Panel D) Effects of beta-estradiol (E2) on miR-140 and Sox2 expression.

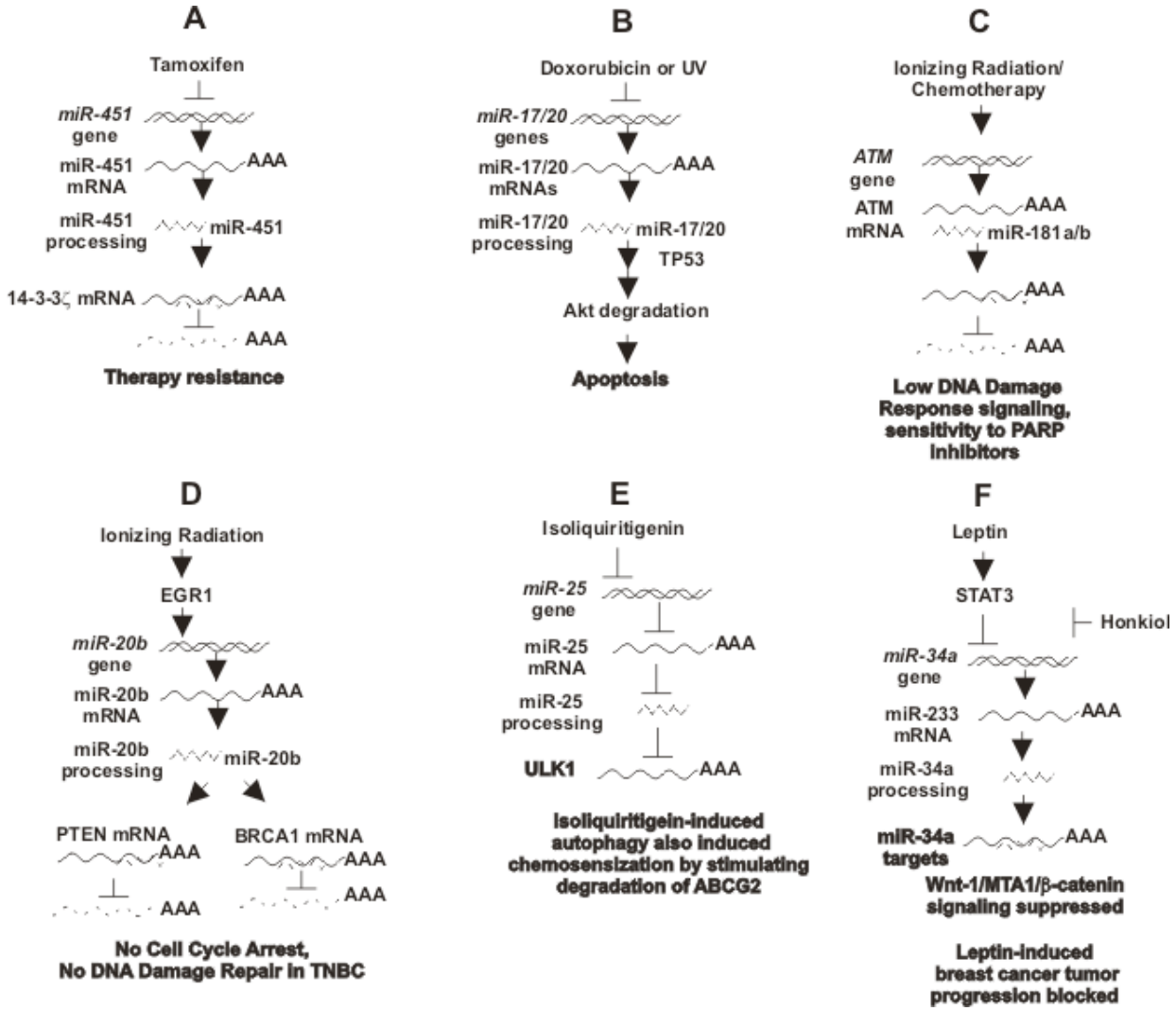

Fig. (5). Effects of drugs and radiation on MIR regulated gene expression. Panel A) Effects of tamoxifen on miR-451 and 14-3-3zeta expression and therapy resistance. B) Effects of either doxorubicin or UV on miR17/20, TP53 and Akt expression, C) Effects of radiation or chemotherapy on ATM and miR-181 expression and sensitivity to PARP inhibitors, D) Effects of ionizing radiation on EGR1, miR-20b, PTEN and BRCA expression in TNBC), E) Effects of isoliquiritigenin on miR-25 and ULK expression and autophagy, F) Effects of leptin on STAT3, miR-34 and Wnt/beta-catenin signaling.

Culturing breast cancer cells with TGF-beta has been documented to increase the ability of the cells to form spheroids which are enriched in CSCs. TGF-beta was determined to modulate miR-181 levels at the post-translational level. Decreased levels of ATM were observed in the cells. Increasing the levels of miR-181 or decreasing the levels of ATM or its substrate checkpoint kinase-2 (CHK2) resulted in more spheroid forming cells. The tumorigenicity of MDA-MB-361 breast cancer cells increased by decreasing ATM expression [230].

There is an oncogenic form of HER2 which is named HER2DELTA16. HER2DELTA16 has been shown to be important in both therapeutic resistance and metastasis. In transfected MCF-7 cells, HER2DELTA16 altered the expression of at least sixteen miRs. One miR that was particularly affected was miR-7m, which was shown to induce a G_1 cell cycle block and to inhibit colony formation. One of the target genes of miR-7 was found to be EGFR. miR-7 suppressed migration in MCF-7/HER2DELTA16 cells by inhibiting EGFR. In contrast, miR-7 suppressed the proliferation of MCF-7/HER2DELTA16 cells by a Src-dependent mechanism. Expression of miR-7 conferred sensitivity of MCF-7/HER2-DELTA16 cells to Herceptin by an EGFR-independent fashion [231].

It was shown that in lapatinib-treated TNBC cancer cells, the HDAC inhibitor trichostatin A induced miR-7. HDAC inhibition can epigenetically suppress EGFR expression. However, in these studies, trichostatin A induced miR-7 in a HDAC-independent fashion. Thus trichostatin A can suppress EGFR expression by other mechanisms besides HDAC inhibition [232].

miR-7 expression was increased by estrogen treatment and suppressed by the antiestrogen fulvestrant (ICI 182 780). miR-7 may be important in hormonal-resistance of certain breast cancer as its expression is blocked by fulvestrant which could result in EGFR expression [233].

Recently it has been shown that miR-141 inhibits EGFR expression. miR-141 and EGFR are also targets of Krüppel-like factor 8 (KLF8) in breast cancer. The dual transcription factor KLF8 has been determined to be important in breast cancer progression. KFL8 and EGFR were determined to be co-overexpressed in invasive breast cancer cells and patient tumor samples. Introduction of KLF8 into non-malignant MCF-10A breast epithelial cells resulted in the induction of EGFR expression. In contrast, knock-down of KLF8 in the TNBC MDA-MB-231 cells reduced EGFR expression. KLF8 was determined to bind directly to the EGFR promoter re-

gion and stimulated EGFR expression. Interestingly KLF8 also bound the miR-141 promoter region and inhibited its expression. miR-141 binds the 3'UTR of EGFR mRNA transcripts to suppress EGFR protein expression. EGFR inhibitors prevented KLF8-induced invasiveness, proliferation *in vitro* as well as invasive growth and lung metastasis in immunocompromised mice. This studies document an important regulatory loop between EGFR, miR-141 and KLF-8 [234].

The role of ER-beta1 has been examined in some basal breast cancer cell lines (MDA-MB-231 and Hs578T). ER-beta1 was shown to repress EMT by destabilizing EGFR by ubiquitylation and degradation. This may occur via the induction of certain miRs including miRs-200a/b and miR-429. Furthermore these miRs may repress ZEB1 and SIF1 expression and result in higher levels of E-cadherin. A positive correlation was observed in basal tumor specimens between ER-beta1 and E-cadherin expression. Induction of EGFR signaling was observed to block the effects of ER-beta1 on preventing EMT. Thus ER-beta1 is important in maintaining the epithelial phenotype of basal breast cells. The authors have proposed that the status of ER-beta1 may be important for determining the extent of metastasis of basal breast cancers [235].

A high-throughput screen was used to identify miRs important in the HER2 pathway in breast cancer. The screen consisted of gain-of-function assays with a miR mimic library and two HER2-amplified cell lines. 38 miRs were identified as important in HER2 signaling. Some of these miRs (*e.g.*, miR-342-5p) were shown to specifically inhibit HER+ but not HER- growth. Elevated expression of this miR was associated with a better survival of both HER2+ and HER2- breast cancer patients. Seven novel miRs targeting HER2 were identified in these studies. These included miR-552, miR-541, miR-193a-5p, miR-453, miR-134, miR-498, and miR-331-3p [236].

miR-133a regulates EGFR expression. It has been determined to be down-regulated in breast cancer cells. Increased expression of miR-133a resulted in decreased EGFR expression, AKT activity and nuclear localization in MCF-7 and MDA-MB-231 cells [237].

Expression of the miR-24 was determined to be higher in breast carcinomas compared to benign breast tissues. Increased expression of miR-24 promoted breast cancer growth, invasion, and metastasis as well as decreased mouse survival. miR-24 targets the tyrosine-protein phosphatase non-receptor type 9 (PTPN9) and receptor-type tyrosine-protein phosphatase F (PTPRF). Increased levels of phosphorylated EGFR were detected in patients with metastatic breast carcinoma which expressed lower levels of PTPN9 and PTPRF [238].

miR-221 and miR-222 have been shown to be important in EMT in breast cancer by increasing the expression of mesenchymal-specific genes and decreasing the expression of epithelial-specific genes in basal breast cancers. The FRA1 transcription factor (FOSL1) was found in basal-like but not luminal breast cancer. FRA1 stimulated the expression of miR-221 and miR-222. miR-221 and miR-222 may exert their effects on EMT and decrease E-cadherin levels by targeting the GATA3 transcriptional repressor family member tricho-rhino-phalangeal syndrome type 1 (TRPS1) which normally serves to suppress ZEB2 expression. Decreased ZEB2 expression normally inhibit EMT [239].

miR-206 and other miRs [miR-18a, miR-18b, miR-22, miR-193b, miR-221/222 and miR-302c] target ERalpha. This can result in the decreased expression of ER target genes [240]. The authors have proposed that the various miRs may have specific functions as well as target other genes.

The selective ER antagonist tamoxifen has been shown to induce the down regulation of miR-451 in breast cancer cells. This can result in elevated 14-3-3zeta expression. High levels of 14-3-3zeta have been associated with earlier disease recurrence. Elevated levels of 14-3-3zeta and decreased levels of miR-451 were observed in tamoxifen-resistant breast cancers [241].

Certain miRs are regulated by c-Myb in breast cancer. A study reported that c-Myb activated the expression of five members of the miR-200 family which were involved in the control of EMT and metastasis [242]. miR-200 activation by c-Myb depends on the expression of the transcriptional repressor ZEB1. During EMT

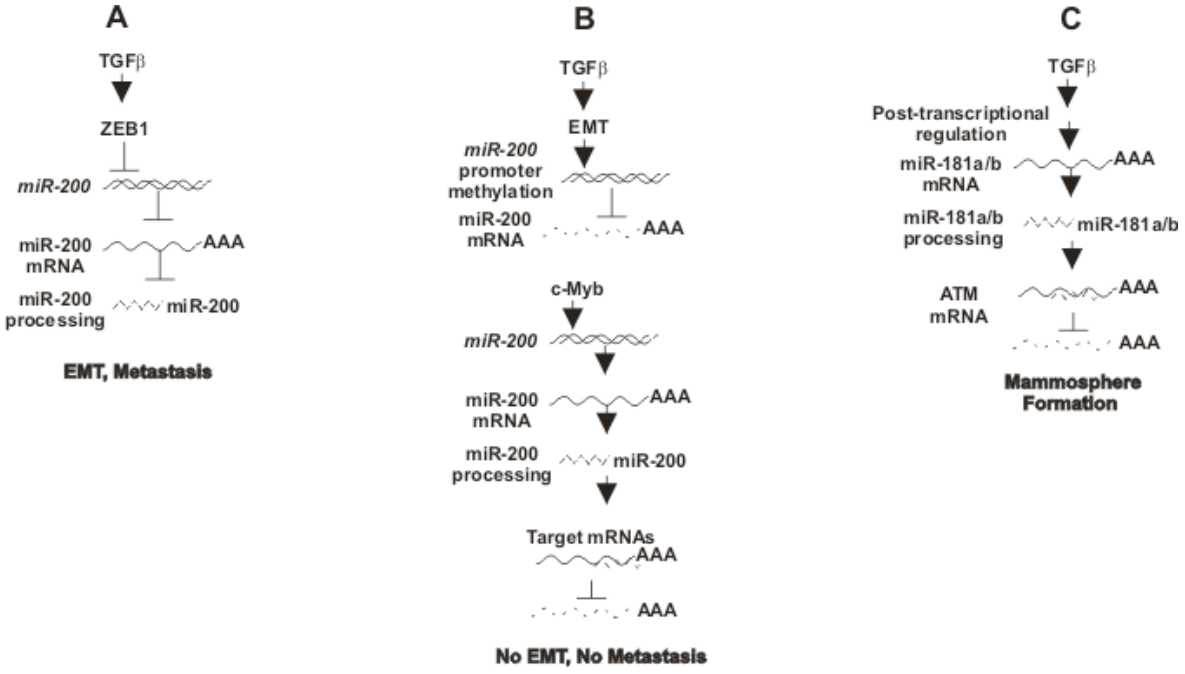

Fig. (6). Effects of TGF-beta and MIRS on EMT, metastasis and mammosphere formation. Panel A) Effects of TGF-beta on Zeb1 and miR-200 on EMT and metastasis, B) Effects of TGF-beta on miR-200 and c-Myb on EMT and metastasis. C) Effects of TGF-beta on miR-181/a/b and ATM and mammosphere formation.

induced by TGF-beta, the promoter regions of miR-200 were methylated. A diagram depicting the interaction between miRs, TGF-beta and EMT is presented in Fig. (**6**).

Certain mutations at *TP53* can result in gain of function (GOF) mutations that alter the effects that TP53 may have on gene expression. Mutant *TP53* genes have been shown to upregulate miRs in breast cancer. Specifically, mutant *TP53* induces upregulation of miR-155 resulting in increased breast cancer metastasis. In this study, an overlap in the targets between mutant TP53 and miR-155 was observed in breast cancers. The *p63* gene normally suppresses a gene that is the target of miR-155. These authors identified the novel zinc-finger transcriptional repressor *ZNF652* as a target gene of miR-155. ZNF652 has been shown to repress the expression of multiple key genes in the TGFbeta, EGFR and vimentin pathways that are important in invasion and metastasis [243]. A diagram depicting the effects of miRs on TP53 and p63 is presented in Fig. (**7**).

The expression of certain miRs may be regulated by ionizing radiation. The promoter region of miR-20b contains an early growth-response consensus binding site and the expression of miR-20b is correlated with early growth response-1 (EGR1) expression. Down regulation of EGR1 suppressed miR-20b in the TNBC cell line HCC 1806. Some targets of miR-20b were identified. These included *PTEN* and *BRCA1* that were down regulated in HCC1806 breast cancer cells. In contrast, suppression of miR-20b increased PTEN and BRCA expression [244].

The *KRAS* oncogene is targeted by miR-200c,a key miR important in EMT and chemoresistance. miR-200c is a potent inhibitor of WT and mutant *KRAS*. Thus miR200c is a suppressor of tumor progression and therapeutic resistance [245]. A diagram depicting the effects of miRs on KRAS is presented in Fig. (**8**).

Kallikrein-related peptidase 5 (KLK5) plays an important role in breast cancer and is sometimes aberrantly expressed. KLK5 normally suppresses malignancy. KLK5 was determined to be down regulated in luminal B and basal breast cancer. Recently it has been shown that KLK5 normally induces miRs associated with an anti-oncogenic pathway in breast cancer. KLK5 expression upregulated 28 miRs and downregulated 62 miRs. Genes encoding extracellular matrix molecules and molecules in cell-adhesion pathways were the most affected [246].

miR-221/222 regulates the expression of alpha6beta4 integrin, an adhesion molecule important in breast cancer progression [247]. One of targets of miR221/miR222 is STAT5a which is normally induced after EGFR activation [5, 248]. miR221/miR222 expression is inversely correlated with tumor growth. In contrast, alpha6beta4 expression was detected at elevated levels in high grade tumors while miR221/miR222 were detected at low levels, suggesting an inverse pattern of expression [247]. miR221/miR222s are involved in the post-transcriptional regulation of STAT5a and ADAM-17. The authors suggested that pre-miR221/miR222 may be a therapeutic strategy in the treatment of aggressive luminal breast cancer subtypes [247].

miR-223 expression has been proposed to be regulated by $p27^{Kip-1}$ which is itself regulated by the EGFR/PI3K/PTEN/Akt/mTORC1 pathway. miR-223 was determined to be upregulated by $p27^{Kip1}$ in G_1-arrested cells. miR-223 expression was deregulated in breast cancer cells but not in normal cells. Contact inhibition was determined to regulate the expression of miR-223 in a $p27^{Kip-1}$ dependent fashion [249]. A diagram depicting effects of miRs on cell cycle progression and drug resistance is presented in Fig. (**9**).

miR-17/20 expression sensitizes breast cancer cells to the induction of apoptosis induced by either doxorubicin or UV irradiation.. miR-17/20 was determined to mediate apoptosis via TP53 which promoted degradation of AKT [250].

The levels of exosomal miRs have been examined in different subtypes of breast cancer patients.MiR-101, miR-372 and miR-373 were detected the most in exosomes. miR-373 levels were higher in circulating exosomes from TNBC and breast cancer patients with luminal carcinomas. Additionally, the authors determined that miR-373 levels were higher in ER- and PR-tumors than hormone-receptor positive tumors. Studies with MCF-7 cells indicated that miR-373 downregulated ER expression and inhibited apoptosis normally induced by camptothecin. Thus these studies suggest that the serum levels of exosomal miR-373 are associated with TNBCs and more aggressive breast carcinomas [251].

The expression of miR-101 is decreased in certain TNBC tissue samples and cell lines. Induced expression of miR-101 inhibited growth and induced apoptosis *in vitro* as well as suppressed tumorigenicity *in vivo*. The anti-apoptotic *MCL1* gene was overex-

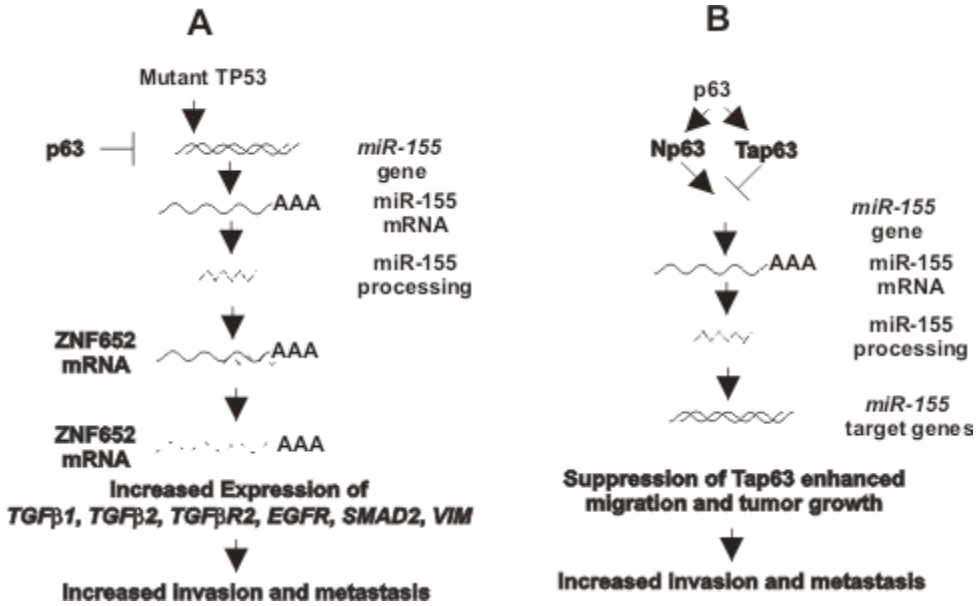

Fig. (7). Effects of MIRS, TP53 and P63 expression and invasiveness and metastasis. Panels A & B) Effects of miR-155 on TP53 and p63 mediated expression of genes involved in invasion and metastasis.

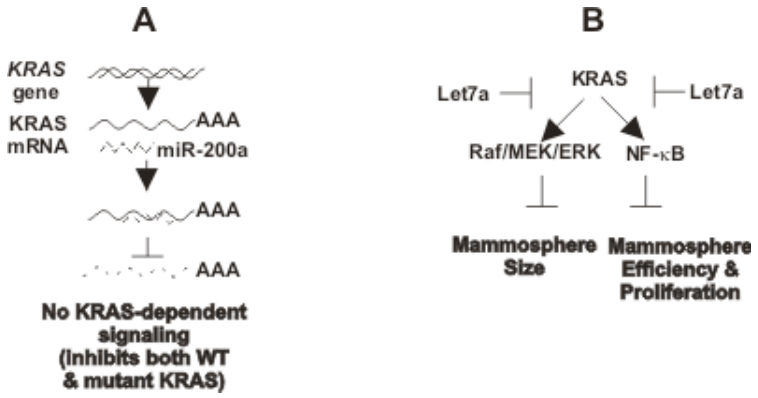

Fig. (8). Interactions between KRAS and MIRS. Panel A) Effects of miR-200a on KRAS gene expression, B) Effects of Let-7a on KRAS-mediated regulation of Raf/MEK/ERK and NF-kappaB and mammospheres.

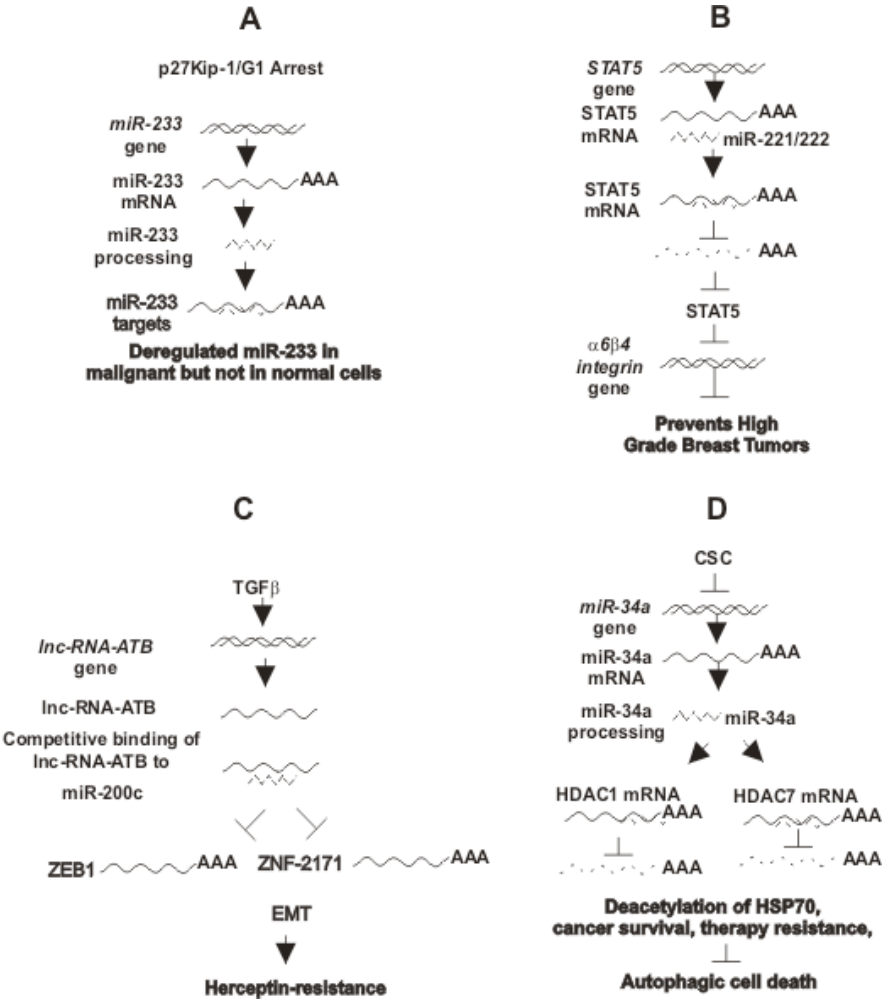

Fig. (9). Interactions between MIRS and LNC-RNAS and genes invovled in resistance and malignant growth. Panel A) Effects of miR-233 on genes involved in cell cycle arrest. B) Effects of miR-221/222 on STAT5a and alpha6beta4 integrin expression and tumor formation. C) Effects of TGF-beta on lnc-RNA-ATB and miR-200C on genes involved in Herceptin resistance. D) Effects of CSC on miR-34a and expression of HDAC genes and therapy resistance.

pressed in the TNBC cell lines and was determined to be a target of miR-101. miR-101 suppressed MCL1 expression. Suppression of MCL1 increased the sensitivity of the TNBC cells to paclitaxel [252]. A diagram depicting interactions of miRs in TNBC is presented in Fig. (**10**).

The presence of miRs in plasma and serum of breast, colorectal, lung, thyroid and melanoma patients and healthy controls was determined by high-throughput technologies. The absolute levels of miR-320a, miR-21-5p, miR-378a-3p, miR-181a-5p, miR-3156-5p, miR-2110, miR-125a-5p, miR-425-5p, miR-766-3p were then determined by droplet PCR. miR-181a-5p was determined to be significantly reduced in breast cancer samples [253].

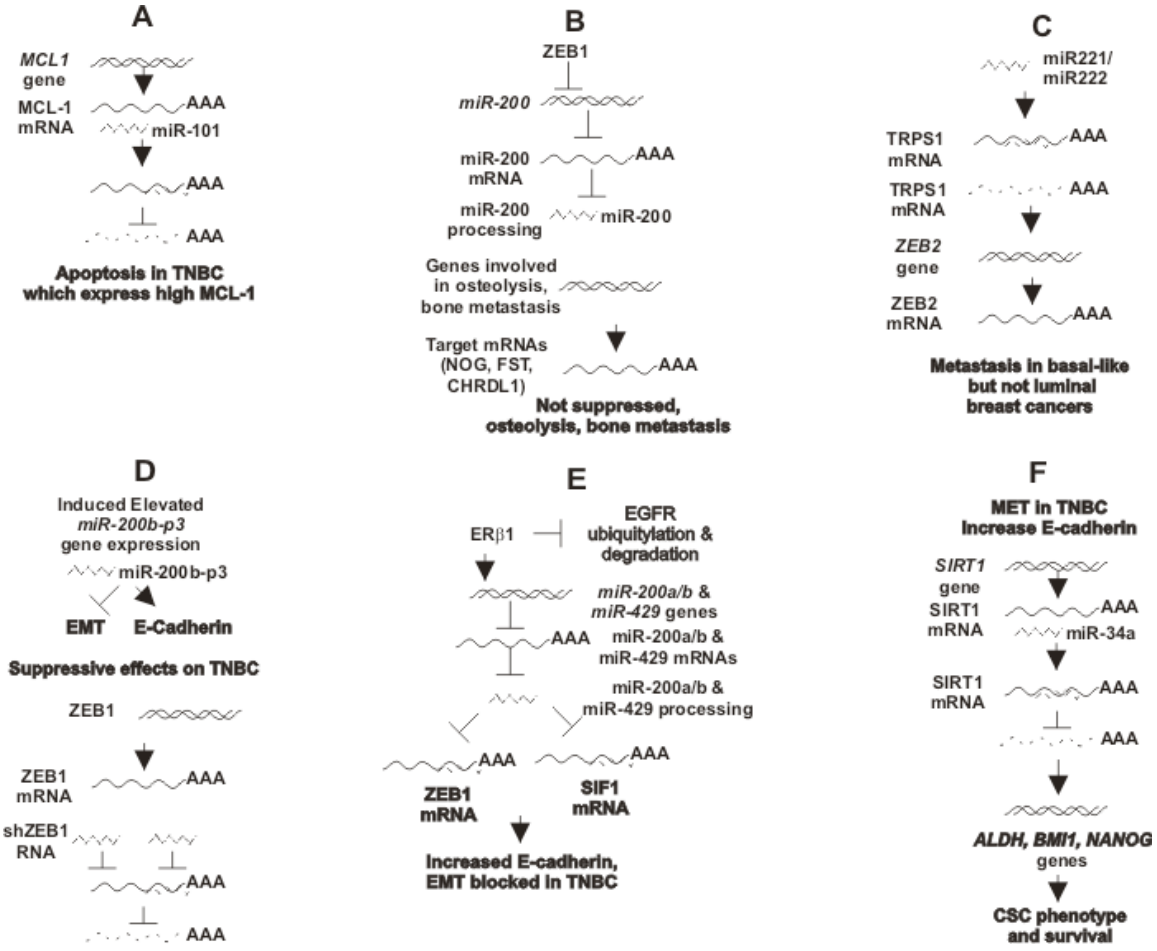

Fig. (10). Effects of MIRS on events invovled in TNBC. Panel A) Effects of miR-101 on Mcl-1 expression in TNBC, B) Effects of Zeb1 on miR-200 expression and genes involved in osteolysis and bone metastasis, C) Effects of miR-221/222 on TRPS1 and Zeb2 gene expression and metastasis of basal-like cancers, D) Effects of miR-200b-p3 on Zeb 1 expression, E) Effects of ERbeta1 on miR-200a/b and miR-429 on Zeb1 and SIF-1 on EMT in TNBC, F) Effects of miR-34a on SIRT1 expression and genes associated with CSCs.

The association of six plasma miRs (miR-16, miR-27a, miR-107, miR-130a, miR-132 and miR-146a) with different types of invasive breast cancers also was examined. In this study, the plasma levels of miRs in 111 breast cancer patients before and after chemotherapy and 46 healthy women were examined by qRT-PCR. miR-16, miR-27a and miR-132 levels were determined to be higher in breast cancer patients before chemotherapy than in healthy women. The levels of miR-27a and miR-132 decreased after chemotherapy to similar levels as observed in healthy women. In contrast the levels of miR-16 did not decrease. Differences in miR levels were also observed between lymph node positive and negative patients and certain miR levels were different between HER+ and HER2- patients. ER- patients exhibited higher levels of miR-107. Thus certain miRs may be important in different breast cancer subsets and may affect tumor progression [254].

Recently the effects of MIA-602, an antagonistic analog of growth hormone-releasing hormone on doxorubicin-sensitive and doxorubicin-resistant TNBC cells, were determined in xenograft studies in the presence and absence of doxorubicin. MIA-602 sensitized both doxorubicin-sensitive and -resistant cells to doxorubicin in the xenograft studies. The response of the cells to doxorubicin, expression of drug resistance genes, and efflux pump function were examined after MIA-602 treatment. MIA-602 lowered the level of growth hormone-releasing hormone and growth hormone-releasing hormone receptors expression. MIA-602 led to decreased expression of ATP-binding cassette (ABC) protein (ABCB1) also known as multi-drug resistance-1 (*MDR1*) which encodes P-glycoprotein (P-gp) and *NANOG* genes as well as efflux pump activity [255, 256]

The *TP63* gene encodes two major isoforms by different promoters (TAp63 and Np63). Tap63 regulates miR-155, an oncogenic miRs upregulated in many solid cancers. Tap63 may prevent the binding of Np63 to the miR-155 gene. Suppression of Tap63 results in elevation of miR-155. This leads to enhanced migration and tumor growth [257].

43 miRs were determined to be differentially expressed in the plasma obtained from breast cancer patients in a study consisting of 122 Caucasian subjects in comparison to healthy controls. miR-148b and miR-133a were observed to be secreted from breast cancer cell lines and may serve as biomarkers for breast cancer detection [258].

p130Cas synergizes with HER2 in mammary cell transformation and promotes HER2-dependent invasion in 3D cultures of human mammary epithelial cells. In the 3D mammary cultures, miR-200b, miR-222, miR-221, miR-210, and miR-424 were found to be upregulated, while miR-27a, miR-27b, and miR-23b were downregulated [259]. miRs have also been shown to be important in breast cancer drug resistance [260].

BRCA1 can regulate the expression of miR-146a which can bind the EGFR mRNA and hence downregulate EGFR expression. If BRCA1 is functional it can increase the levels of miR-146a that

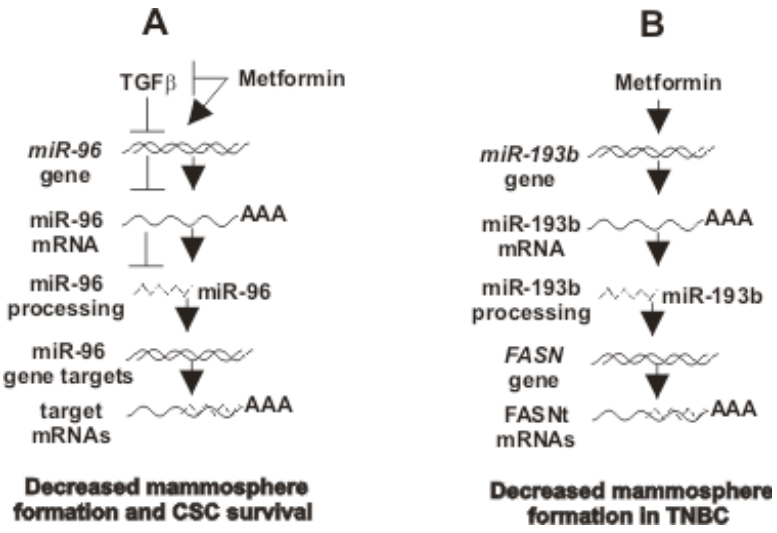

Fig. (11). Effects of metformin on MIR expression and mammosphere formation. Panel A) Effects of metformin on miR-96 and mammosphere formation, B) effects of metformin on miR-193b and FASN expression and mammosphere formation.

in turn decreases the levels of EGFR. BRCA1 can bind to the promoter region of miR-146a and regulate its expression. *BRCA1* is frequently mutated in certain cancer families and breast cancer patients. Thus loss of functional *BRCA1* could lead to increased EGFR expression. These studies also observed a positive correlation between BRCA1 and miR-146a expression in TNBC patient samples. Furthermore low miR-146a expression was determined to predict positive lymph node status [261].

The expression of let-7a miR was downregulated in early stages of breast cancer while the expression of KRas was upregulated in early and advanced stages of breast cancer. Overexpression of let-7a miR has been shown to inhibit proliferation and mammosphere formation in a KRas- dependent fashion. let-7a exerted it effects on mammosphere formation efficiency by the NF-kappaB pathway andelicited its effects on mammosphere-size by the MAPK/ERK pathway [262]. Table **1** summarizes the effects of certain regulatory RNAs on breast cancer.

PIWI interacting small non coding RNAs (piRNAs) are important in RNA silencing and gene regulation. Their expression has been determined to be different in breast cancer cells as opposed to mammary epithelial cells. Their expression in breast cancer may be influenced by estrogen and ER-beta expression [263].

Long non-coding RNAs (lncRNAs) are also important in breast cancer. The mechanisms by which lncRNAs regulate gene expression in different breast cancer subtypes are under investigation. lncRNA HOX Antisense Intergenic RNA (HOTAIR) was determined to be overexpressed in the HER2 subgroup, while the lncRNA HOTAIRM1 was overexpressed in the basal-like breast cancers. The authors have proposed that lncRNAs may influence cancer through increased activity of neighboring genes [264].

Treatment of TNBC cells with the EGFR/HER2 inhibitor lapatinib and the c-Abl inhibitor imatinib has been shown to have some efficacy and synergy in pre-clinical models of breast cancer. This is believed to occur by suppression of the lncRNA HOTAIR. HOTAIR is normally regulated by beta-catenin via a lymphoid enhancer-binding factor 1 (LEF1)/TCF4-binding site. Co-treatment with lapatinib and imatinib was shown to prevent the nuclear expression of beta-catenin and inhibited the binding of beta-catenin to the HOTAIR promoter [265].

lncRNAs have also been demonstrated to be involved in Herceptin-resistance. Herceptin-resistant cells were generated and they were determined to have elevated TGF-beta signaling as well as more cells that had undergone EMT. The lncRNA-ATB was activated by TGF-beta in both Herceptin-resistant SKBR-3 cells and tissues derived from Herceptin-resistant patient cells. Interestingly the lncRNA-ATB was shown to induce Herceptin-resistance and an invasion/metastasis cascade in breast cancer cells. This occurred by the lnc-RNA-ATB binding miR-200c and inducing ZEB1, Zinc Finger Protein 217 (ZNF-217) and EMT. ZNF-217 is prognostic biomarker and therapeutic target in breast cancer [266].

CCAT2 is another lncRNA. CCAT2 expression was examined in 997 primary breast cancer patients. CCAT2 expression was found to be informative for lymph node positive disease which had received adjuvant cyclophosphamide, methotrexate, and fluorouracil (CMF) chemotherapy. CCAT2 promotes cell migration and reduces sensitivity to 5-fluorouracil [267]. The expression of the LINC00472 lncRNA has been examined in breast tumor samples and was associated with a good prognosis [268].

A take home message for this section is that regulatory RNAs play critical roles in breast cancer. The number of regulatory RNAs and their gene targets is constantly expanding,.. Identification of methods to target regulatory RNAs is clearly an emerging and important area in breast cancer research and therapy.

THERAPEUTIC RESISTANCE IN BREAST CANCER INDUCED BY EGFR AND DOWNSTREAM SIGNALING PATHWAYS

Both the Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways lie downstream of the EGFR family members. These pathways are frequently implicated in breast cancer drug resistance as well as drug resistance in other types of cancer [269-274].

We often think of these pathways in relatively simplistic terms, mainly that they are linear and flow downstream from EGFR and other receptors. However these pathways are also regulated by additional kinases, phosphatases, scaffolding molecules and other proteins. Furthermore there are intricate feedback loops which serve to regulate their activities. Many of these other proteins can also influence these pathways and the sensitivity to targeted, hormonal-based, radio- and chemotherapy as well as therapy resistance [275-281].

Increased expression of activated ERK has been observed in tamoxifen-resistant breast cancer. Treatment of MCF-7 cells with

Table 1. Effects of miRs on Gene Expression in Breast Câncer miRs Which Target EGFR.

miRNA	Gene Modulator/Target	Effect	References
miR-7	EGFR	G1 cell cycle block, inhibits colony formation	[231]
miR-7	EGFR	Induced by trichostatin A	[232]
miR-7	Inhibited by fulvestrant	EGFR expression, therapeutic resistance	[233]
miR-133a	EGFR	Suppresses downstream Akt signaling	[237]
miR-141	EGFR	Invasion and proliferation	[234]
miR-146a	EGFR	BRCA1 induces miR-146a	[261]
miR-566		Activates EGFR pathway signaling	[37]
miRs Organized in Numerical Order			
miR-7	EGFR	G1 cell cycle block, inhibits colony formation	[231]
miR-7	EGFR	Induced by trichostatin A	[232]
miR-7	Inhibited by fulvestrant	EGFR expression, therapeutic resistance	[233]
miR-17/20	Induced by doxorubicin or UV	Regulation of apoptosis by TP53 and degradation of Akt	[250]
miR-18a, miR-18b, miR-22, miR-193b, miR-206, miR-221/222 and miR-302c	ERalpha	Altered expression of ER target genes.	[240]
miR-20b	PTEN, BRCA1, radiation	Sensitivity to therapy	[244]
miR-21	ZEB1	Migration of TNBC	[204]
miR-24	PTPN9 & PTPRF	Increased EGFR activity, invasion, metastasis	[238]
miR-25		Regulation of chemoresistance and autophagy induced by isoliquiritigenin	[309]
miR-27a, miR-132		Higher in breast cancer patients after chemotherapy	[254]
miR-34a	Wnt-1/MTA1/beta-catenin signaling	Honokiol inhibited Leptin induced tumor progression via miR-34a	[310]
miR-34a	SIRT1	Inhibits stemness in breast cancer	[313]
mIR-101	MCL-1	Inhibits TNBC growth and increases sensitivity to paclitaxel	[252]
miR-125a	LIFR/Hippo	Breast CSC pool	[225]
miR-133a	EGFR	Suppress downstream Akt signaling	[237]
miR-140	Sox2	E2 enhances CSC survival by downregulation of miR-140	[293]
miR-141	EGFR	Invasion and proliferation	[234]
miR-146a	EGFR	BRCA1 induces miR-146a	[261]
miR-155	Mutant p53 inducer, ZNF652 target	Increased invasion	[243]
miR-155	Tap63 may prevent binding of Np63 to miR-155 promoter	Suppression of Tap63 results in enhance migration and tumor growth	[257]
miR-181a/b	ATM	Aggressive breast cancers	[229]
miR-181	ATM	Spheroid formation	[230]
miR-193b	FASN	Metformin induced miR-193b and death of TNBCs	[331]
miR-200	ZEB1	Induction of EMT, bone metastasis associated genes	[221]

(Table 1) Contd….

miRNA	Gene Modulator/Target	Effect	References
miR-200	Regulated by ZEB1, c-Myc and promoter methylation	EMT and metastasis	[242]
miR-200b-3p & miR-200b-5p		Suppression of EMT in TNBC, increase in E-cadherin	[220]
miR-200a/b & miR-429	ZEB1 & SIF1	Higher levels of E-cadherin in basal breast cancer cells	[235]
miR-200c	KRas	Tumor progression, therapeutic resistance	[245]
miR-221 & miR-222	Increase mesenchymal genes, decrease epithelial gene	EMT, decreased E-cadherin	[239]
miR-221/222	Alpha6beta4 integrin	Adhesion molecule important in breast cancer progression	[247]
miR-223	p27Kip-1 regulates in G1 arrested cells	Regulation in contact-inhibited cells but deregulated in breast cancer cells.	[249]
miR-451	14-3-3zeta	Sensitivity to tamoxifen	[241]
Let-7a	KRas	Mammosphere size and proliferation	[262]
Let-7a	Metformin induced let-7a, suppressed TGFbeta induced miR-181 and downregulation of miR-96	Metformin suppressed TGFbeta induced mammosphere formation	[330]
Snc-RNAs			
PIWI	Expression influenced by E2 and ERbeta	Transcriptional and post-transcriptional gene regulatory actions	[263]
Lnc-RNAs			
HOTAIR		Overexpressed in HER2 cancers	[264]
HOTAIRM1		Overexpressed in basal-like cancers	[264]
HOTAIR	Regulated by beta-catenin	Sensitivity to lapatinib and imatinib treatment of TNBC	[265]
lncRNA-ATB	Activated by TGF-beta in Herceptin-resistance cells, binds miR200c and activates ZEB1, ZNF-217	Involved in EMT, Herceptin resistance, invasion and metastasis	[266]

tamoxifen increased the levels of MAPK kinase phosphatase-2 (MKP-2) but not MKP-1 protein levels. Enhanced expression of MKP-1 or MKP-2 suppressed E2-induced proliferation. MKP-2 was determined to increase sensitivity to tamoxifen while MKP-1 overexpression did not. ERK1/2 phosphorylation was lost in MKP-1 and MKP-2 overexpressing cells. Interestingly in tamoxifen-resistant MCF7-TAMR cells, higher levels of MKP-2 mRNA and protein were detected as well as activated ERK1 and ERK2 proteins. Thus MKP-2 may play a role in promoting sensitivity of cells to tamoxifen [282].

The MAPK-interacting kinases (MNK) regulate eIF4E phosphorylation that is important in protein translation and may be a novel approach to treat HER+ and TNBCs. Recently retinoic acid metabolism blocking agents (RAMBS) retinamides (RRs) have been evaluated in TNBC and HER2+ breast cancer cells. RRs induce the degradation of MNK1 in TNBC and HER2+ cells which blocks eIF4E phosphorylation resulting in suppression of growth, colonization, invasion, and apoptosis. The effects of the RRs are mediated by MNK degradation rather than inhibition of its kinase activity [283].

Many diverse genetic mechanisms have been associated with resistance to various therapeutic approaches. The leucine zipper tumor suppressor gene-1 (LZTS1) product alters cell mitosis by the stabilization of microtubule networks. The *LZTS1* gene is located on chromosome 8p22 which is frequently altered in human cancer. It has been shown recently that down regulation of LZTS1 expression causes resistance to taxanes, microtubule-stabilizing drugs. The expression of LZTS1 was examined in 270 primary breast cancer patient samples. LZTS1 expression was determined to be down-regulated, associated with both a higher incidence of tumor reoccurrence, worse overall survival and unfavorable outcome after taxane therapy [284].

The AT-rich interactive domain-containing protein 1A (ARID1A) has helicase and ATPase activities. The ARID1A may regulate transcription of certain genes by modifying the chromatin structure around certain target genes. ARID1A mutations may confer a synthetic lethal interaction with PI3K/Akt inhibition in breast and other cancer types [285].

Certain PARP inhibitors may sensitize breast and other cancers to radiation therapy. Niraparib (MK-4827), is a novel PARP inhibitor that radio-sensitized breast and other cancers to radiation which was independent of their *TP53* gene status. In contrast, it did not exert these effects on cells derived from normal tissues. The authors suggested that the mechanism might involve the conversion of sin-

gle strand breaks into lethal double strand breaks during DNA replication. This may occur by niraparib inhibiting base excision repair [286].

NF-kappaB expression is associated with doxorubicin-resistance in breast cancer. Defects in *TP53* are necessary for the transcriptional activation of NF-kappaB target genes that doxorubicin can induce. A *TP53*-deficient background was determined to correlate with the NF-kappaB-dependent gene expression signature: intercellular adhesion molecule 1 (*ICAM1*), chemokine (C-X-C motif) ligand 1 (*CXCL1*), tumor necrosis factor, alpha-induced protein-3 (*TNFAIP3*), and *IL8*. The presence of a TP53-deficient background in breast cancer patients was associated with a reduced disease-free survival [287].

Targeting HER2 with lapatinib inhibits stem/progenitor proliferation. HER2 is highly expressed in 20% DCIS and is also expressed in CSCs. Lapatinib was shown to not reduce self-renewal in the CSCs but inhibited their proliferation regardless of their *HER2* status. These important studies indicate that lapatinib can lower DCIC CSC activity and suggest that lapatinib has potential therapeutic usefulness in preventing recurrence and progression of DCIC to invasive disease [288]. The efficiency of Herceptin is dependent on its ability to target HER2 in HER2-driven CSCs. However, the CSCs can develop resistance to Herceptin. EMT may be important in regulating the sensitivity to Herceptin [289].

Elevated PI3K pathway activity and low TNFalpha and IFNgamma signaling were found to be part of the genetic signature for high risk tumors in a HER2+/ERalpha- mouse model (MMTV-Her2/Neu). The HER2+/ERalpha- tumors had a TIC genetic signature [290].

A take home message for this section is that therapeutic resistant can result from the abnormal expression of many different genes. This number of molecules involved and potential drug targets are rapidly expanding due to improved genomic and proteomic approaches.

STEMNESS AND REPROGRAMMING IN BREAST CANCER

The mTOR pathway is important in the nuclear reprograming of MCF-7 cells. Upon transfection of MCF-7 cells with the stemness factors: OCT-4, SOX2, Kruppel-like factor 4 (KLF4), rare colonies with an induced pluripotent stem cell (iPSC) state were obtained. These cells were named MCF-7/Rep cells and the authors concluded that they represent an intermediate state between cancer cells and *bona fide* iPSCs. The MCF-7/Rep cells overexpressed endogenous SOXs but silenced the exogenous *SOX2* gene. The SOX-overexpressing MCF-7/Rep cells contained higher levels of CD44 and ALDEFLOUR-stained ALDH (bright) cells than MCF-7 cells. The gene expression patterns of MCF-7/Rep cells were found to be distinct from those of the MCF-7 cells. Interestingly they displayed elevated insulin receptor (IR) gene expression, upregulation of p70S6K, and increased phosphorylation of mTORC1 in the SOX2-overexpressing CSC-like population. Thus the mTORC1 pathway is implicated in the generation of CSCs and targeting this pathway may be an appropriate therapeutic approach for certain breast cancers [291].

SOX2 is a prognostic marker for the detection of early recurrence in breast cancer. In a study of 117 breast cancer patients, NANOG, GDF3 and SOX2 mRNA expression correlated with grade 2, node-negative and proliferation rates. SOX2 expression was associated with increased risk of recurrence as well as node status [292]. E2 may increase CSC activity by the SOX2 stemness factor and enhance CSC survival by down regulation of miR-140 that normally targets SOX2 [293].

ERalpha suppresses EMT and factors involved in stemness in human breast cancer. The polycomb group protein B lymphoma Mo-MLV insertion region 1 homolog (Bmi1) has been shown to regulate EMT and maintain the self-renewal capacity of stem cells. Recently it has been shown that ERalpha increased E-cadherin expression by downregulation of Bmi1 in breast cancer cells. Overexpression of ERalpha also suppressed migration, invasion, and EMT of breast cancer cells. ERalpha overexpression also suppressed the CD44high/CD24low population and the capacity for mammosphere formation in ERalpha- breast cancer cells. Overexpression of Bmi1 inhibited ERalpha suppression of EMT and stemness. In human breast cancer tissue, an inverse relationship was observed between ERalpha and Bmi-1 expression [294].

4EGI-1 is an inhibitor of the eukaryotic translation initiation factor 4E1 (eIF4E1) and eIF4G1. 4EGI-1 suppressed breast CSCs by reducing protein translation. Importantly, 4EG1-1 suppressed the translation of NANOG, OCT4, CXCR4, c-Myc and VEGF in breast CSCs. Many of these proteins are important in stemness. These studies document the importance of selective inhibition of translation by 4EGI-1 in breast CSCs [295].

The high mobility group A1 (HMGA1) proteins play key roles in the metastasis of basal-like breast cancers. Knockdown of HMGA1 induced mesenchymal to epithelial transition (MET), decreased stemness, self-renewal, migration, invasion and metastasis. HMGA1 induced stemness and genes associated with migration that were linked to the Wnt/beta-catenin, Notch, and peptidyl-prolyl cis/trans isomerase-1 (Pin1) pathways [296].

Surgery is the only curative intervention in breast cancer. However, surgery can induce inflammatory responses. The wound fluids (WF) from surgery patients are rich in cytokines and growth factors that can induce the STAT transcription factor and stimulate the growth of breast cancer cells. Furthermore the WF promoted the enrichment of breast cells with stem-like properties. Inhibiting STAT3 suppressed this process. These studies point to the initial involvement of the microenvironment and breast CSCs [297].

Gene products involved in acyltransfer may be important in the breast cancer microenvironment. The 1-acylglycerol-3-phosphate O-acyltransferase 9 (AGPAT9) was expressed at higher levels in the poorly invasive MCF-7 cells than in highly invasive MD-MBA-231 cells. AGPAT9 was shown to inhibit invasion and proliferation [298].

There are few effective treatments for TNBC. Up to 20% of women with TNBC under the age of 50 contain germline mutations in *BRCA1* and these patients may be sensitive to PARP inhibitors. However, the other 80% of TNBC patients lack *BRCA1* mutations. The effects of a pan-histone deacetylase inhibitor (HDI) were examined on TNBC cells lacking *BRCA1* mutations. The HDI induced "BRCAness" in the TNBC cells which lacked *BRCA1* mutations and conferred sensitivity to either the PARP inhibitor ABT-888 or cisplatin. Combining HDI and ABT-888 induced more DNA strand breaks than either drug alone and also resulted in more apoptosis and reduced tumor growth in mouse models and improved survival [299].

Cyclin D1 and its partner CDK4/6 play key roles in cell cycle progression in both normal and malignant cells. Targeting cyclin D1 and CDK4/6 had effects on the migration and breast cancer stem-like activity. Inhibition of cyclin D1 and CDK4 expression with specific siRNAs had different effects depending on ER expression. Inhibition of their expression suppressed migration and stem-like activity in ER+ but enhanced migration and stem-like activity in ER- breast cancers. Treatment with inhibitors of cyclin D1 and CDK4/6 (flavopiridol/PD0332991) yielded similar results as obtained with the siRNAs for cyclin D1 and CDK4/6 [300].

Many EGFR+ breast cancer patients are also positive for markers associated with EMT. Activation of EGFR signaling by its ligand on MCF-7 breast cancer cells was sufficient to induce the EMT phenotype, inhibit apoptotic events and mediate the loss of cytokeratin expression [301]. Some of these events are associated with CSCs and drug resistance.

The relationship between the cell cycle, migration and stem cell like activity has been examined in breast cancer by live cell sorting based on the phase of the cell cycle, expression of stem cell markers (CD44/CD24/ESA), migration and ability to form mammospheres. An inverse relationship between proliferation and migration and mammosphere formation was observed. Cells in G_0/G_1 exhibited increased migration and mammosphere formation. A subpopulation of low proliferative stem-like cells (CD44+/CD24low/ESA+) was identified which had increased migration and mammosphere formation. This population was specifically inhibited by Dickkopf1 (DKK1) and dibenzazepine (DBZ) which are known to inhibit stem cells [302].

P-cadherin is an important cell-cell adhesion molecule that has a role in breast cancer progression. P-cadherin confers invasive properties to basal-like breast cells. Inhibition of P-cadherin decreased cell adhesion to laminin and reduced expression of the laminin receptor alpha6 beta4 integrin as well as mammosphere formation. P-cadherins were shown to regulate many molecules including the alpha6beta4 integrin, Fak, Src and Akt. Thus P-cadherin and its downstream components could be targets for breast cancer therapy [303].

A take home message for this section is that many different types of molecules are being shown to be involved in breast cancer stemness and nuclear reprogramming. These molecules have different functions, from existing as cell surface receptors to transcription factors which control the expression of subsets of genes involved in stemness and reprogramming. In addition, gene products involved in signaling, cell cycle progression and apoptosis are also involved in stemness and reprogramming. Some of the gene products represent targets of drugs which are often used to treat other diseases and not normally associated with breast cancer therapy.

EFFECTS OF NUTRACEUTICS AND DRUGS USED FOR OTHER DISEASES ON BREAST CSCS

Sulforaphane is a natural compound derived from broccoli/broccoli sprouts. It has been shown to suppress mammosphere formation of breast CSCs *in vitro* as well as tumor formation in mice studies with breast CSCs [304].

The effects of the soy isoflavones were examined in patient samples taken prior to treatment as well as after treatment by NanoString technology [305]. This study determined that there were some differences in gene expression observed in the patients who had the soy supplements. Genes whose expression changed included genes involved in cell cycle progression and proliferation, such as fibroblast growth factor receptor 2 (*FGFR2*), E2F transcription factor 5 (*E2F5*), the mitotic checkpoint serine/threonine-protein kinase budding uninhibited by benzimidazoles (*BUB1*), Cyclin B2 (*CCNB2*), v-Myb avian myeloblastosis viral oncogene homolog-like 2 (*MYBL2*), cyclin dependent kinase-1 (*CDK1*), and cell division cycle 20 (*CDC20*). The addition of soy isoflavones to the diet of breast cancer patients remains controversial.

Garcinia cambogia is a fruit often used in weight loss, especially with type II diabetes. The Utilization of the caged *Garcinia* xanthone (CGX) motif may be an approach to inhibit spontaneously-forming spheroids. Spheroids are often implicated in CSCs. This compound has been shown to inhibit spheroid formation in the spheroidsMARY-X, 3-dimensional multicellular system [306].

The natural phenol isoliquiritigenin (ISL) is a licorice chalconoid that has been shown to have pronounced effects on breast CSCs [307]. It is a sirtuin-activating compound. Sirtuins remove acetyl groups from proteins. Recently a novel function of ISLs has been identified. It can be a demethylation agent. ISL enhanced WNT inhibitory factor 1 (WIF1) expression which inhibited breast cancer and reduced CSC-like cells. ISL treatment also resulted in decreased expression of beta-catenin and G_0/G_1 arrest of CSCs. ISL enhanced WIF1 expression by inducing the demethylation of the WIF1 promoter region. Inhibiting WIF1 prevented the effects of ISL on breast CSC levels. ISL may be able to bind the catalytic domain of the DNA (cytosine-5-)-methyltransferase 1 (DNMT1) and alter its activity [308].

miR-25 regulates chemoresistance and autophagy induced by isoliquiritigenin in breast cancer [309]. Isoliquiritigenin induced chemosensitization, cell cycle arrest and autophagy in drug resistant cells. miR-25 was determined to be the main target of isoliquiritigenin in triggering the autophagy flux. Inhibition of miR-25 resulted in autophagic cell death by increasing Unc-51-like kinase-1 (ULK1) expression. ULK1 is an early regulator in the induction of autophagy. Isoliquiritigenin had chemosensitization effects and resulted in the downregulation of ABC protein ABCG2 by an autophagy-lysosome pathway. These studies indicate the potential of isoliquiritigenin in breast cancer therapy and reveal the role of miR-25 as a regulator of autophagy by targeting ULK1. The EGFR/PI3K/PTEN/Akt/mTORC1 pathway can also regulate ULK1.

Obesity is believed to influence the risk, progression and prognosis of many cancers, including breast cancer. The effects of obesity are mediated to a large extent by the adipocytokine leptin. Honokiol (HNK) is a bioactive polyphenol isolated from *Magnolia grandiflora* and has been shown to have effects on leptin-mediated breast cancer growth. HNK was determined to inhibit the leptin-induced Wnt-1-metastasis-associated protein 1 (MTA1)-beta-catenin signaling via miR-34a. HNK inhibited STAT3 phosphorylation which prevented STAT3 recruitment to the miR-34a promoter region which normally serves a suppressor of miR-34a. miR-34a was thus activated and could inhibit Wnt-1-MTA1-beta-catenin signaling [310].

HER2 can also associate with integrins, such as integrin alpha 6 (ITGA6) and integrin beta 4 (ITGB4), which are also overexpressed in certain breast cancers. The medicinal plant derived cucurbitacin (CuB) derived from *Hemsleya endecaphylla* and other plants (*e.g.*, *Cucurbita andreana*), inhibited the expression of ITGA6 and ITGB4. Moreover CuB induced the expression of ITGB1 and ITGB3 that can cause integrin-mediated cell death. TGF-beta treatment resulted in increased association of HER2 with ITGA6. CuB treatment could prevent this association. These studies indicate an approach to treat breast cancer by targeting HER2 and integrins with curcurbitacin and other drugs [311].

HIF-1alpha induced resistance to endocrine therapy in ER+ breast cancers. Detection of HIF-1alpha before treatment was associated with a negative clinical outcome. Treatment with the aromatase inhibitor letrozole resulted in increased levels of HIF-1alpha compared with pre-treatment levels. The Ras/Raf/MEK/ERK pathway may be involved in the induction of HIF-1alpha transcription as treatment with zoledronic acid suppressed Ras/Raf/MEK/ERK pathway and HIF-1alpha expression. Zoledronic acid is used in the treatment of postmenopausal osteoporosis. These studies indicate the role of HIF-1alpha and Raf/MEK/ERK pathways in the development of certain endocrine therapies and the potential of zoledronic acid to be used in breast cancer patients who have failed certain endocrine therapies [312].

Sirtuin-1 (SIRT1) has been shown to be important in stemness in breast CSC. miR-34a regulates SIRT expression. High and low levels of SIRT1 and miR-34a were observed respectively in CD44+/CD24- breast CSCs and manipulating their expression was determined to effect mammosphere formation. Increased expression of miR-34a and decreased expression of SIRT1 resulted in decreased expression of ALDH1, BMI1 and Nanog as well as reduced tumor burden in xenograft studies [313].

Flubendazole is a FDA-approved drug used for the treatment of intestinal parasites. Flubendazole has recently been shown to have anti-proliferative effects on breast cancer CSCs. In addition flubendazole delayed tumor growth in xenograft models. Flubendazole

was determined to reduce the CD44high/CD24low subpopulation and inhibit mammosphere formation. Flubendazole also decreased the expression of certain genes associated with stemness including *CCND1* (cyclin D1), *MYC*, *NANOG*, *SOX2* and *OCT4* and the expression of mesenchymal markers including beta-catenin, N-cadherin and vimentin. Flubendazole arrested the cells at the G_2/M phase of the cell cycle. Flubendazole was determined to augment the cytotoxic activity of chemotherapeutic drugs such as doxorubicin and 5-fluoruracil [314].

Disulfiram is a drug used to treat certain alcoholic patients. DSF has been shown to target TNBC CSCs and reverses resistance to paclitaxel. The MDA-MBA-231PAC10 TNBC line is resistant to multiple chemotherapeutic drugs including paclitaxel, cisplatin, docetaxel and doxorubicin. MDA-MBA-231PAC10 cells have a longer doubling time and are more quiescent than MDA-MB-231 cells. The drug resistant MDA-MBA-231PAC10 cells were found to express numerous markers associated with CSCs including ALDH1, Oct4, Sox2, NANOG and displayed nuclear localization of HIF-2alpha and NF-kappaB (p65). DSF treatment eliminated the expression of these markers often associated with stemness and CSCs and rendered the MDA-MBA-231PAC10 cells sensitive to certain chemotherapeutic drugs including paclitaxel and cisplatin [315].

One of the problems of radiation therapy (RT) in the treatment of breast cancer is that RT may induce radio-resistant CSCs. RT may induce the expression of genes associated with stemness. RT may also stimulate the reprogramming of differentiated breast cancer cells into CSCs by inducing the re-expression of stemness genes. Interestingly, targeting the NF-kappaB pathway with DSF and copper can block RT-induced stemness. DSF inhibits ALDH. DSF binds copper which inhibits the proteasome and NF-kappaB activation. This was shown to inhibit primary mammary tumors in mice [316].

Breast CSCs have been shown to have an enhanced ability to utilize catabolic sources of energy under starvation circumstances. It has been suggested that metabolic reprogramming may be an adaptive strategy by which CSCs survive during nutrient deprivation. These studies point to the importance of metabolic reprogramming during cancer progression [317]. Other authors have also presented models on the importance of glycolysis and tumor metabolism [318].

Elevated glucose uptake is a property of many metastatic tumors. This can result from mutant oncogenes. Activation of the Ras pathway increases glycolytic flux into lactate. The hepatocyte growth factor (HGF) binds the Met tyrosine kinase receptor that in turn activates Ras and downstream ERK. This resulted in increased motility and glucose uptake. This has been shown to regulate the metabolism of certain breast cancers as well as influence blood-flow in the tumors [319].

New mitochondrial biogenesis has been shown to be important in breast CSC. XCT790 is a specific inhibitor of ER-related alpha-peroxisome proliferator-activated receptor gamma coactivator 1 (ERRalpha-PGC1) signaling. This pathway is important in mitochondrial biogenesis. XCT790 has been shown to block the survival of MCF-7 TICs. XCT790 suppressed the activities of Sonic hedgehog, TGFbeta-SMAD, STAT3 and Wnt signaling. Overexpression of ER-alpha enhanced the efficiency of mammosphere formation which could be blocked by the addition of various mitochondrial inhibitors including doxycycline that is an FDA-approved antibiotic [320].

Metformin (*N,N*-Dimethylimidodicarbonimidic diamide) has been shown to have effects on various CSC, including both breast and pancreatic cancers to name a few. Some of the effects are mediated by the regulation of miR expression, others are modulated by the activation of signaling pathways mediated by AMP kinase (AMPK) [321].

The effects of metformin and the related biguanide phenformin were examined in a Src-inducible model of transformation as well as in mammosphere-derived breast CSCs by metabolomics in an attempt to understand how these drugs preferentially affect CSCs. During the process of cellular transformation, treatment with the biguanides suppressed the increase of glycolytic intermediates and decreased tricarboxylic acid (TCA) intermediates at certain stages of transformation. In contrast, the effects on glycolytic and TCA intermediates were not as pronounced in breast CSCs. However, reductions in nucleotide triphosphates levels were observed which could result in suppression of nucleotide biosynthesis. The authors proposed that the biguanides inhibited mitochondrial complex 1 and their metabolic effects may differ at various stages of the transformation process which is important in breast cancer progression [322].

Metformin may be able to induce metabolic reprogramming of chemoresistant breast cells. Metformin was determined to induce metabolism in ALDH-bright but not ALDH-low cells. Metformin treatment affected glutathione metabolism in the ALDH-bright cells. Metformin altered miR expression in ALDH-bright cells. Many of the affected miRs were believed to target metabolic pathways. Thus metformin may reduce the differences between the chemoresistant ALDH-bright and chemosensitive ALDH-low cells [323]. Many pathways involved in regulation and signal transduction may also be important in regulation of inflammation and cancer [324].

Resistance to metformin in breast cancer can result in a metastatic stem-like phenotype. Resistance to metformin resulted in the expression of many genes encoding components of the degradome, cancer cell migration, invasion factors, stem cell markers and pro-metastatic lipases. In addition, downregulation of genes involved the G_2/M DNA damage checkpoint regulation that maintain genome stability as well as alteration of gene expression with pro-autophagic features were observed [325, 326].

The effects of metformin and hyperthermia were examined on MCF-7 and MDA-MB-231 breast and MIA PaCa-2 pancreatic cancer cells. Metformin was determined to be preferentially cytotoxic to CD44high/CD24low MCF-7 cells and CD44high/CD24high Mia-PaCa-2 cells. The CD44high/CD24low and CD44high/CD24high phenotypes are associated with breast and pancreatic CSCs respectively. Heating at 42^0C for one hr. enhanced the ability of metformin to kill cancer cells as well as CSCs. Hyperthermia was determined to activate AMPK and inactivate mTORC1 and downstream p70S6K. Thus hyperthermia could enhance the cytotoxic effects of metformin on both breast and pancreatic CSC [327].

Metformin treatment has been shown to increase the radiosensitivity of certain radio-resistant CSCs. RT and metformin treatment both activated AMPK that resulted in inactivation of mTORC1, p70S6K and 4EBP1 [328].

The carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), bisphenol A (an organic compound used in the manufacture of many things including plastic water bottles) and E2 all induce OCT-4 expression as well as the size and number of MCF-7 mammospheres. OCT4 expression was monitored as a CSC marker. In contrast, metformin decreased the number and size of MCF-7 mammospheres. Metformin also reduced the expression of OCT4 in mammospheres induced by E2 and TCDD but not in mammospheres induced by bisphenol-A [329].

The expression of 88 cancer-related miRs was examined in MCF-7 cells in the presence and absence of metformin. Metformin was determined to increase the expression of lethal-7a (Let-7a) miR in MCF-7 cells and suppressed the TGF-beta increased miR-181a expression which resulted in enhanced mammosphere formation ability. MCF-7 cells induced to undergo EMT in response to TGF-beta increased miR-181a expression and displayed enhanced mammosphere formation. Metformin prevented this increase in miR-

181a expression. Metformin blocked the ability of TGF-beta to increase mammosphere formation and the ability of TGF-beta to down-regulate miR-96, a tumor suppressor miR. These studies indicate how metformin may be able to prevent EMT-regulated CIC self-renewal [330]. Fig. (**11**) presents a depiction of the effects of metformin on various miRs.

Metformin also has been shown to reduce the expression of fatty acid synthetase (FASN) in TNBC by inducing miR-193b. miR-193b was shown to be induced by metformin and bind the 3'UTR of FASN. The effects of miR-193b were observed in TNBC but not in non-transformed breast epithelial cells. Metformin was shown to induce miR-193 which reduced mammosphere formation in TNBC cell lines [331].

Recently it has been proposed that metformin is synthetically lethal with glucose withdrawal in breast CSC. This may be due to the CSCs having increased dependency on Warburg-like aerobic glycolysis to maintain CSC stemness and immortality that is eliminated upon metformin treatment. Under conditions of glucose-deprivation, metformin inhibited the ability of oncogenes such as HER2 to prevent apoptosis [332].

Metformin by itself was shown to inhibit mammosphere formation in SKBR3 parental Herceptin-sensitive cells, SKBR3 TzbR (Herceptin resistant cells) and JIMT-1 (a line resistant to Herceptin and other HER2-targeted therapies). In contrast, Herceptin reduced mammosphere formation in SKBR3 cells but not in SKBR3 TzbR or JIMT-1 cells. Metformin and Herceptin treatment decreased mammosphere formation ability in Herceptin-resistant cell lines which overexpressed CD44 (CD44+/CD24low/neg). In these studies, the CD44-overexpressing Herceptin-resistant SKBR3 and JIMT-3 cells were very sensitive to treatment with metformin by itself. However combining Herceptin with metformin induced a synergistic response in suppressing HER2+ mammosphere formation efficiency and self-renewal in both SKBR3 TzbR and JIMT-1 cells. Metformin was shown to selectively kill CD44+/CD24-/low cells purified from the Herceptin-resistant JIMT-1 breast cancer cell line to treatment with Herceptin. The CSC isolated from the JIMT-1 cells were more sensitive to Herceptin and metformin treatment than the non-CSC JIMT-1 cells [333].

Metformin has been shown to inhibit the CD61 high, CD49f high TIC subpopulation in the murine mammary tumor virus (MMTV)-ErbB2 (HER2) mammary tumor model. Metformin treatment resulted in reduced tumor development in syngeneic tumor transplant models. The expression of ErbB2 (HER2) and EGFR1 proteins were decreased after metformin treatment and their levels of phosphorylation also decreased. In addition, metformin treatment resulted in the decreased phosphorylation of IGF-1R, Akt, mTOR and STAT-3. Metformin was determined to inhibit self-renewal/proliferation of CSC/TICs in ErbB2-overexpressing cells. High levels of ErbB2 were detected in breast CSC/TIC-enriched tumorspheres. These studies highlight the important roles of ErbB2 (HER2) in breast CSC/TICs and document the possibility of using metformin to target these cells [334].

There are at least 33 clinical trials with the anti-type II diabetes drug metformin and breast cancer [335]. Some of these clinical trials are examining the effects of combining metformin and EGFR/HER2 inhibitors (erlotinib and lapatinib), rapalogs (everolimus, sirolimus, temsirolimus), statins (atorvastatin), aromatase inhibitors (exemestane), chemotherapeutic drugs (docetaxel, epirubicin, myocet, cyclophosphamide, doxorubicin), omega-3 fatty acids and antibodies against the IGF-1R (ganitumab).

Recently the results of a clinical trial examining the effects of combining the aromatase inhibitor exemestane, metformin and the peroxisome proliferator-activated receptor gamma (PPARgamma) agonist rosiglitazone were investigated in 20 breast cancer patients. Rosiglitazone activates PPARgamma receptors and results in redistribution of fat away from viscera and liver. Rosiglitazone also improves insulin sensitivity. In this small clinical trial, the doses of the three drugs were well tolerated and six patients (30%) had stable disease for six months. Metformin did not appear to alter the pharmacological properties of exemestane [336].

A take home message for this section is that nutraceuticals and drugs used in the treatment of other diseases are being investigated as potential supplemental approaches to treat or to prevent the development of breast cancer. Some of these potential treatments include drugs that are currently clinically used in the therapies of other diseases (e.g., diabetes, alcoholism and others) and are approved for treatments of those diseases. In some cases the effectiveness of some of these potential treatments may be obtained by analysis of the effects of their usage in the long term treatment of other diseases and determination if the patients developed breast cancer, especially if the patient belonged to a family with a history of breast cancer.

ANDROGEN RECEPTOR AND BREAST CANCER

A breast cancer subset also expresses the androgen receptor (AR), HER2, EGFR but often lacks ER expression. These breast cancers are referred to as apocrine carcinomas [337]. Recently five different subtypes of TNBC have been proposed. Classification of TNBC into different subtypes might improve or allow personalized TNBC therapy. The five subclasses proposed are: 1) TNBC with deficiencies in DNA repair or growth factor signaling pathways and have basal properties, 2) TNBC with cells having undergone EMT and processing CSC and mesenchymal properties, 3) TNBC associated with immune properties, 4) TNBC with AR overexpression and 5) TNBC that are HER2 enriched [338].

The expression of the HER2 was examined in relations to the hormonal receptors (ER, PR, AR), EGFR, TP53, and Ki67 was examined by quantum dot (QD)-based nanotechnology in 240 invasive breast cancer patients. Patients could be divided in two subtypes of HER2+, high and low HER2, and three types of hormone receptor+, high, low and no receptors. Similar to other, earlier studies, a negative correlation was observed between HER2 and hormone receptor expression. 46.3% of the breast cancer samples were positive for AR expression. EGFR expression was associated with 5-year disease free survival rates in those patients that were hormone receptor or lymph node positive [339].

Molecular apocrine (MA) breast cancers are characterized as being ER- but AR+. The expression of many markers was examined in 58 MA tumors by qRT-PCR. The MA tumors were AR+ and 93% were ER-. 67% of the MA tumors were HER2(3+). MA tumors were determined to be clinically aggressive [340].

In a recent study, higher AR expression was observed in ER+ (57.8%) and in ER- (24.7%) breast cancer samples. In this study, it was observed that in the ER+ cancer patients, AR expression was associated with better clinicopathological features. In contrast, AR expression was associated with different parameters in ER- breast cancer tumors which had features of MA cancers. AR+,MA cancer patients had a better overall survival than AR-,MA cancer patients [341].

In another study, samples from 83 TNBC cancer patients, the expression of the ER, HER2, AR, EGFR and other markers were examined. 32.5% of the TNBC samples were positive for AR. In this study, an association between AR staining and disease free or overall survival was not observed [342]. Similarly, in a study with 119 cases of TNBCs breast cancer samples, higher expression of the autophagy markers nuclear nucleoporin (p62) and beclin-1 were observed in the MA than other TNBCs. These observations suggested that there was higher autophagy activity in the MA subset of TNBCs [343].

The MAPK pathway was shown to be important in the growth induced by AR signaling in ER- human breast cancers. MAPK activation resulted in $p21^{Cip-1}$ induction and was required for the

response to AR activation. This was determined in MCF-10A and MDA-MB-231 cells that were transfected with a full length AR gene. The requirement for $p21^{Cip-1}$ was determined in AR- transfected MCF-10A cells which had $p21^{Cip-1}$ knocked-out. These studies determined that MAPK activation from both EGFR and AR hyper-activation in the AR-transfected cells resulted in an inhibitor response. In contrast, activation of either AR or EGFR by themselves leads to MAPK activation which resulted in cell growth. The responses to AR on MAPK activation were determined to be dependent on the presence of $p21^{Cip-1}$ [344].

In TNBC, EGFR expression is frequently detected in both apocrine and non-apocrine tumors [345]. The expression of EGFR and HER2 were examined in a group of 55 apocrine carcinomas. It was determined, in this study, that HER2 and EGFR expression were inversely correlated. Amplification of the *EGFR* gene was observed in two pure apocrine carcinomas and one apocrine-like carcinoms. Polysomy of chromosome 7 was present in 61% of the pure apocrine carcinomas. The *EGFR* gene is located on chromosome 7 [346].

Upon gene expression analysis of chemoresistant TNBC patient samples, it was observed that the TNBC subgroup, which was predicted to have a good prognosis, had high expression of "luminal-like" genes including AR and GATA3. In contrast in the subgroup with the worse prognosis, expression of genes associated with CSCs was observed [347].

A take home message for this section is that certain types of-breast cancer express the AR. TNBCs are historically difficult to treat. This AR+ subclass of TNBC may be sensitive to drugs which target the AR.

CONCLUSION

Because the EGFR family of receptors is frequently overexpressed in breast cancer, these receptors are critical therapeutic targets. EGFR is frequently overexpressed in TNBC and associated with a poor prognosis. There are few therapeutic options for TNBC. Moreover, this review has also discussed how key signaling pathways, such as PI3K/PTEN/Akt/mTOR and Ras/Raf/MEK/ERK, are frequently deregulated in breast cancer due the abnormal expression of EGFR family members as well as mutations in key components of these signaling pathways. Inhibitors of EGFR family members have been developed and some are used to treat breast and other cancers and some may even be used to inhibit breast CSCs. Some of these inhibitors may be combined with other inhibitors or drugs used to treat other diseases. Relatively novel mechanisms of gene regulation have recently been shown to influence the expression of key genes involved in breast cancer. These include miRs and lncRNAs. These may represent novel markers and targets for therapy.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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