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Review

Signaling pathways in breast cancer: Therapeutic targeting of the microenvironment

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ABSTRACT

Breast cancer is the most common cancer in women worldwide. Understanding the biology of this malignant disease is a prerequisite for selecting an appropriate treatment. Cell cycle alterations are seen in many cancers, including breast cancer. Newly popular targeted agents in breast cancer include cyclin dependent kinase inhibitors (CDKIs) which are agents inhibiting the function of cyclin dependent kinases (CDKs) and agents targeting proto-oncogenic signaling pathways like Notch, Wnt, and SHH (Sonic hedgehog). CDKIs are categorized as selective and non-selective inhibitors of CDK. CDKIs have been tried as monotherapy and combination therapy. The CDKI Palbocyclib is now a promising therapeutic in breast cancer. This drug recently entered phase III trial for estrogen receptor (ER) positive breast cancer after showing encouraging results in progression free survival in a phase II trials.

The tumor microenvironment is now recognized as a significant factor in cancer treatment response. The tumor microenvironment is increasingly considered as a target for combination therapy of breast cancer. Recent findings in the signaling pathways in breast cancer are herein summarized and discussed. Furthermore, the therapeutic targeting of the microenvironment in breast cancer is also considered.

Contents

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1. Introduction

Cell division and cell death are the two predominant physiological processes that regulate normal tissue homeostasis. Alteration of these two physiological processes has a pivotal role in the pathogenesis of cancer [1], a disease that consists of immortal cells which can be fatal for patients. Great efforts to ascertain components of the cell cycle are guiding to novel approaches for the treatment of cancer.

Genes encoding components of the cell cycle such as cyclin, CDKs and their endogenous inhibitors which are found in normal conditions are often impaired in many human cancers [2]. For example, CDKs are overactive in some cancers depending on cyclin overexpression or downregulation of endogenous CDKIs [3]. According to this data, researchers focus on whether the strategy of CDK inhibition is able to render cancer treatment more successful. Some studies suggest that inhibiting CDKs may be an effective therapy in many cancers including breast cancer [4]. Hormone therapy is a systemic therap most often used as an adjuvant to reduce the risk of cancer relapse after surgery. It is also used to treat cancer that has come back after treatment or has spread. Breakthrough in clinical oncology offered the possibility of expanding the ways patients with breast cancer are treated with hormone therapy by using drugs that block estrogen from binding to their receptors on tumor cells, preventing cells from growing and spreading [5]. Some pathways such as Notch, Wnt, SHH (Sonic hedgehog) and other pathways have recently been reported as a novel therapeutic target in breast cancer [6–10].

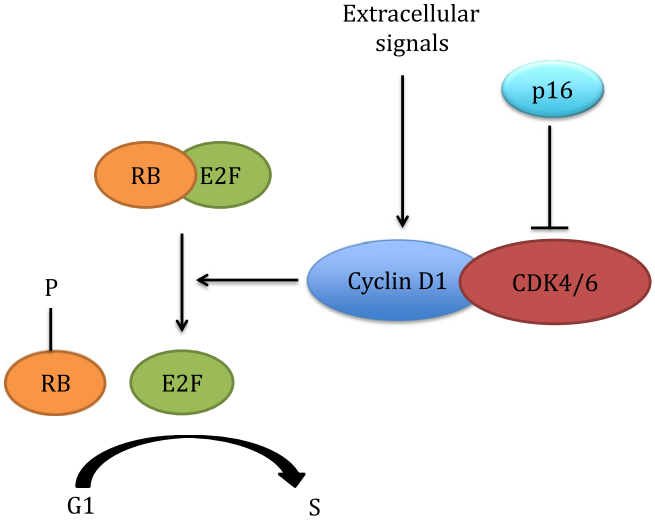

Fig. 1. Proteins, pathways in breast cancer cell cycle and survival: Cyclin D1/CDK4 and CDK6/Rb/E2F pathway for G1 to S transition [16–18].

The breast microenvironment consists of extracellular matrix (ECM) and numerous stromal cell types, including endothelial and immune cells, fibroblasts, and adipocytes [11]. Recent studies have reported that cancer-associated fibroblasts (CAFs), which make up the bulk of cancer stroma and tumor microenvironment, promote cancer initiation, angiogenesis, invasion, and metastasis [11]. In breast cancer, CAFs not only promote tumor progression but also induce therapeutic resistance. Accordingly, targeting CAFs provides a novel way to control tumors with therapeutic resistance [11]. Breast tumor cells express some Notch molecules and release factors that promote cancer cells survival and proliferation [12–14]. The tumor microenvironment is now recognized as an important participant of tumor progression and response to treatment [15]. Consequently, there is increasing interest in developing novel therapies targeting the microenvironment, particularly as it relates to invasiveness and metastatic progression. Signals from the microenvironment, especially those from transforming growth factor-β (TGF-β), induce targeted de novo epigenetic alterations of cancer-related genes [15]. TGF-β signaling has been reported two opposite roles in cancer, namely tumor suppression and tumor promotion, and its deregulation is at least partly induced by epigenetic alteration [15]. The present review summarizes and discusses the current understanding of the signaling pathways in breast cancer with a particular emphasis on the therapeutic potential of microenvironment targeting.

2. Signaling pathways and hormones involved in breast cancer cell cycle and survival

Several proteins, pathways and hormones are involved in breast cancer cell cycle and survival such as CDKs (Cyclin dependent kinase), Notch, Wnt, SHH, estrogen receptor, HER2 (human epidermal growth factor receptor 2), and others. Fig. 1 show proteins, pathways in breast cancer cell cycle and survival.

2.1. CDKs

Cell cycle is regulated by cyclins, CDKs, and CDKIs. These three key classes of regulatory molecules determine a cell's progress through the cell cycle [16]. Cell cycle is divided into 4 distinct phases (G1, S, G2, and M). G0 represents exit from the cell cycle. Specific cyclin and CDK complexes conduct cell cycle progression by regulating transition through G0-G1-S-G2-M phases. Cell cycle is driven by CDKs, which are positively and negatively regulated by cyclins and CDKIs, respectively [17]. Cyclins form the regulatory subunits and CDKs the catalytic subunits of an activated heterodimer; cyclins have no catalytic activity and CDKs are inactive in the absence of a partner cyclin [18].

Animal cells contain lots of CDKs. Some of them are directly involved in cell cycle regulation, such as CDK1, CDK2 and CDK4. For example, CDK1, with its partners cyclin A2 and B1, alone can drive the cell cycle

in mammalian cells [19]. When activated by a bound cyclin, CDKs perform a common biochemical reaction called phosphorylation that activates or inactivates target proteins to orchestrate coordinated entry into the next phase of the cell cycle. Cyclin–CDK complexes in earlier cell-cycle phase help activate cyclin–CDK complexes in later phases [20]. In addition, a second group of CDKs are responsible for the regulation of cellular transcription. They have role of maintenance for cancer cells' survival. This group of CDKs consists of CDK7, CDK8, CDK9, CDK10, and CDK11.

A CDKI protein is an endogenous protein that interacts with a cyclin–CDK complex to block kinase activity, usually during G1 or in response to signals from the environment or from damaged DNA. In the human body, there are two major CDKI protein families: the INK4a/ARF family and the Cip/Kip family. INK4s, which are strictly inhibitory, bind CDK monomers. Evidence from the study of crystal structures of CDK6–INK4 complexes show that INK4 binding twists CDK6 to distort cyclin binding and kinase activity. On the other hand, Cip/Kip proteins bind both cyclins and CDKs of complexes, resulting in either inhibitory or activating effects. The Cip/Kip family proteins activate cyclin D and CDK4 or CDK6 complexes by enhancing complex formation [21].

To push the cell from G1 to S phase the phosphorylation of retinoblastoma (Rb) protein by CDK4 or CDK6 in complex with their activating subunits, cyclin D1, D2 and D3 is necessary. The hyperphosphorylated Rb protein dissociates from the E2F/DP1/Rb complex to activate E2F. Activation of E2F results in transcription of various genes such as cyclin E, cyclin A, DNA polymerase, and thymidine kinase. For instance cyclin E thus produced binds to CDK2, forming the cyclin E–CDK2 complex that keeps up the progression through G1-S phase. CDK2-cyclin A and CDK1-cyclin A regulate the completion of S phase. Then G1/S progression initiates the G2/M transition [22]. Finally, the cell cycle is completed and cell is going to divide.

All cancers activate cell cycle to sustain their survival. Selecting an appropriate agent for the appropriate tumor type is very hard, because, first of all, it should be identified which regulator of the cell cycle is responsible for the cell cycle downstream of an oncogenic event. Therefore, mouse models have been used to understand what kind of the cell cycle inhibitor is against which cancer type. In many cancers CDKs are overactive or CDK-inhibiting proteins are dysfunctional. For example, upregulation of CDK4 or downregulation of a naturally occurring inhibitor of CDK4, called p16INK4A, lead to loss of proliferative control of cell through enhanced CDK4 activity, resulting in hyperphosphorylation of Rb protein and in carcinogenesis [23]. According to this information, it is rational to target CDK function to prevent over proliferation of cancer cells and to use CDKIs to treat human cancers.

2.2. Notch signaling

2.2.1. Notch ligands and receptors

The Notch signaling pathway has been implicated in the pathogenesis of breast cancer and as such may represent a novel therapeutic target. Notch signaling consist of 5 Notch ligands, Delta-like (Dll) 1, 3, 4, and Jagged (JAG) 1, 2, which are single transmembrane proteins, containing a characteristic extracellular DSL domain that mediates receptor binding, and multiple EGF-like repeats. Jagged ligands have an extra cysteine-rich domain, which is not present in the Delta-like ligands. The cytoplasmic regions of these ligands are not well characterized except for the C-terminal domain that contains a PDZ-binding motif [24–27].

There are 4 Notch transmembrane receptors: Notch 1–4, which are synthesized individually from independent mRNAs as single protein precursors that undergo glycosylation by the enzyme protein O-fucosyl transferase in the endoplasmic reticulum. Some of the O-fucose moieties are further elongated by Fringe glycosyltransferases (Lunatic, Manic, and Radical), which modify the specificity of the receptor for its ligand [28]. Notch receptors are then cleaved by the protease furin in the trans Golgi network into 2 non covalently linked domains, the notch extracellular domain and the notch intracellular domain (NICD). The notch extracellular domain contains a variable number of epidermal growth factor (EGF)-like repeats (between 26 and 29 depending on the Notch receptor); 3 LNR (LIN12/Notch related) repeats, which prevent ligand-independent signaling), and 2 conserved cysteine residues. The C-terminal transactivation domain contains a PEST sequence that facilitates rapid proteolytic degradation of the protein.

All 4 Notch receptors use the same basic signaling pathway that is activated by binding of Notch ligand on one cell to the extracellular domain of a Notch receptor on a neighboring cell. The Notch ligand–receptor complex then undergoes several key proteolytic cleavages. The cleavage is initially mediated by the ADAM/TACE family of proteases and occurs at an extracellular site (S2), between Ala (1710) and Val (1711) residues, approximately 12 amino acids outside the transmembrane domain. This generates a product known as NEXT (notch extracellular truncation), which is then cleaved by the γ-secretase complex, which consists of 2 key proteins, presenilin and nicastrin. Presenilin is the catalytic component of γ-secretase, whereas nicastrin is not catalytically active but promotes the maturation and proper trafficking of other proteins in the complex [29]. γ-Secretase cleaves NEXT, which is the critical step that releases NICD that translocates into the nucleus and associates with CSL [CBF-1 (C-promoter binding factor 1), Suppressor of Hairless and Lag-1], a constitutive transcriptional repressor [30–33] (Fig. 2). After Notch binding, CSL becomes a transcriptional activator and, in conjunction with cofactors such as mastermind-like (MAML) proteins, induces transcription of downstream targets including several Hairy/Enhancer of Split related genes (Hes, Hey), pTa, and Notch1 itself [34,35]. Both Hes and Hey proteins contain a basic domain, which determines DNA binding specificity and a helix–loop–helix domain, which allows for the formation of homo- or heterodimers. Either by interacting with co-repressors or by sequestering transcriptional activators, dimers of hes and/or hey proteins regulate the transcription of key genes [36]. These transcriptional targets include cell-cycle regulators (p21 and cyclin D1), transcription factors (c-Myc, NF-Kb2), and growth factor receptors (HER2) and regulators of angiogenesis and apoptosis [37–43] (Fig. 2). Disruption of the Notch pathway can therefore have significant downstream effects on cell growth, differentiation, angiogenesis, and apoptosis.

2.2.2. Notch signaling and tumorigenesis

The first indication that Notch plays a role in tumorigenesis came from the identification of the t(7:9)(q34; q34.3) chromosomal translocation in a subset of human pre-T-cell acute lymphoblastic leukemias (T-ALL). This translocation resulted in a truncated and constitutively active Notch 1 receptor under the control of the T-cell receptor beta promoter (TCR-B). Subsequently, activating mutations in Notch 1 have been discovered in more than 50% of human T-ALL cases [44,45]. Abnormalities in various components of the Notch pathway have also been found in solid tumors [46–49].

In murine mammary cancers, the Notch 4 locus is a common proviral integration site for the MMTV (mouse mammary tumor virus), which induces mammary adenocarcinomas [50]. MMTV insertion results in constitutive, ligand-independent expression of Notch 4 ICD and increased activation of Notch target genes. Human breast cancer cell lines have also been tested for Notch expression: A truncated and activated form of Notch 4 has been found in 2 of 8 cell lines and an activated Notch 1 ICD in 8 of 8 cell lines tested [51,52]. Notch 3 seems to play a role specifically in the proliferation of Erb2-negative breast cancer cell lines [53].

Studies in primary human breast cancers have shown that high-level expression of Jag1 ($Jag1^{High}$) and/or Notch1 ($Notch1^{High}$) mRNA in tumors correlates with poor outcome and is an independent prognostic indicator [54–56]. It has also been shown that NUMB, a key negative

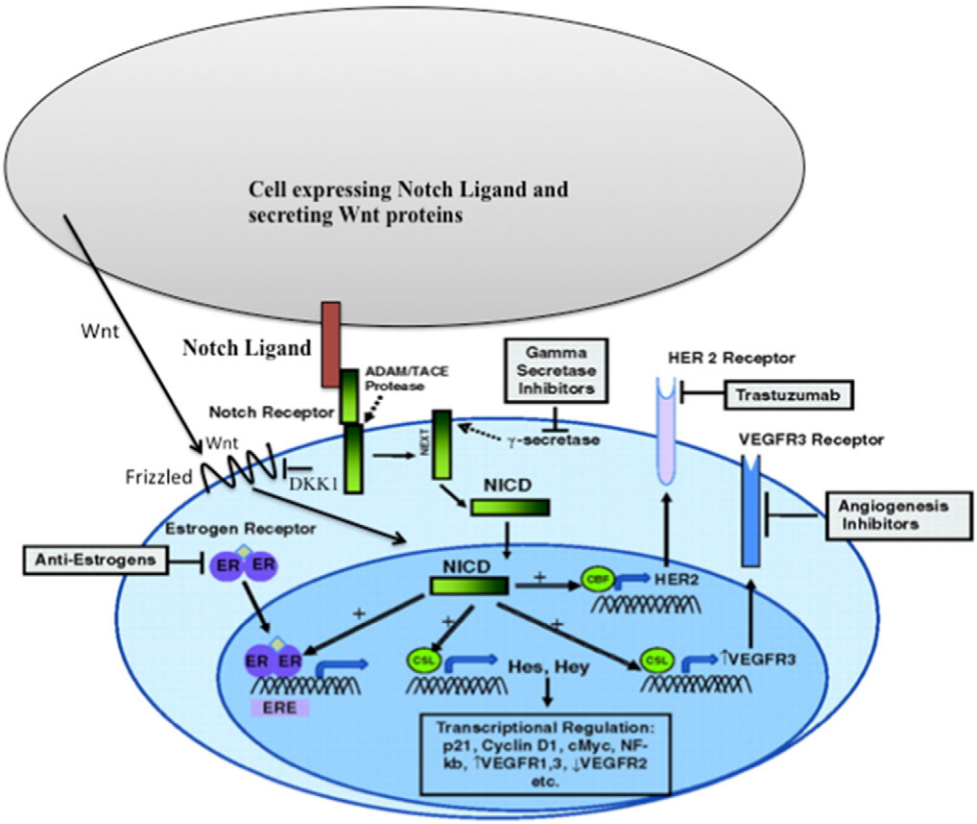

Fig. 2. The membrane-tethered Notch receptor is activated by binding to a ligand on a neighboring cell. This binding results in an initial cleavage triggered by the ADAM17/TACE metalloprotease, resulting in the generation of the next product, which is further cleaved by γ-secretase, resulting in the release of the intracellular domain of Notch (NICD). NICD translocates to the nucleus, causing transactivation of downstream target genes including several genes (Hes, Hey). A number of signaling pathways may interact with Notch in the transformation of breast epithelial cells. These include the ER pathway, signaling downstream of Her2 and the VEGFR. Pharmacologic inhibitors of these pathways in combination with GSI are being tested in the context of breast cancer [30–33]. Aberrant activation of Wnt signaling has been described in a number of human cancers including colorectal cancer, ovarian cancer and breast cancer [83,88]. β-catenin expression has been associated with poor prognosis in breast cancer patients in a number of studies [89,90] and is enriched in basal-like breast cancer [87]. Furthermore loss of negative pathway regulators such as the extracellular inhibitor of Wnt signaling, secreted Frizzled-related protein 1 (*sFRP1*), is found in many breast tumors and is associated with poor prognosis [84,89]. Down regulation of the inhibitor Dickkopf 1 (*DKK1*) in a lung metastases derived MCF7-LM cell line demonstrates the importance of Wnt regulation in the metastatic process in breast cancer [92].

regulator of the Notch pathway, is lost in greater than 50% of tumors due to ubiquitination and proteosomal degradation, and this also correlated with higher grade tumors [57].

2.2.3. Notch signaling and cross talk

The oncogenic role of Notch in breast cancer may be mediated in part through its cross talk with other signaling pathways, such as the estrogen pathway. Approximately 80% of breast cancers express the estrogen receptor and are treated with anti-estrogens, but resistance to anti-estrogens often develops. One mechanism of resistance may be via the Notch pathway. In the absence of estrogens, Notch signaling becomes activated and can directly stimulate estrogen receptor a-dependent transcription, overriding the inhibitory effects of anti-estrogens [58]. From a therapeutic standpoint, concurrently targeting both the estrogen receptor and the notch pathway may help to overcome or at least in part delay this resistance.

The Notch pathway may also interact with the human epidermal growth factor receptor 2 (HER2) signaling pathway, which is active in approximately 20% of breast cancers and associated with a more aggressive disease. CBF-1 (which forms a complex with NICD) has been shown to have binding sequences in the HER2 promoter and at the same time inhibition of Notch signaling seems to downregulate Her2 expression. Taken together, this suggests an important link between these 2 pathways [59]. Using HER2-targeted agents, such as trastuzumab or lapatinib in combination or in sequence with Notch pathway inhibitors, may therefore be a strategy that warrants further study.

Notch is also involved in angiogenesis, which is critical for tumor growth and proliferation. Zeng et al. have shown that Notch signaling from tumor cells can trigger Notch activation of neighboring endothelial cells and promote tumor angiogenesis [60]. Notch signaling itself seems to increase levels of vascular endothelial growth factor receptors (VEGFR1, 3; VEGFR3 the upstream promoter of VEGFR3 contains Notch-responsive CSL elements) and decreases VEGFR2 expression [61–63] (Fig. 2). Conversely, VEGF may also directly regulate expression of the Notch ligand Dll4 in tumor vessels. It has been shown that Dll4 levels correlate with VEGF levels and VEGF blockade results in a rapid and profound reduction of Dll4 expression [64,65]. In a small study of 19 patients with metastatic breast cancer treated with 1 dose of the antiangiogenic agent bevacizumab, biopsies taken before and after treatment show increased expression of both VEGF and Notch target genes (hes and hey), again supporting an interaction between these 2 pathways [66]. Concurrently, targeting both the notch pathway and the angiogenic pathway could therefore be explored further as long as toxicity is not a major problem. Notch may also cooperate with the ERK pathway [67]. Constitutively active Notch 1 requires the ERK pathway to mediate transformation of immortalized breast cells, and activated Notch-positive tumors expressing phopsho-Erk1/2 in the nuclei showed high node positivity. This suggests that Notch–Erk cooperation may not only be necessary for disease progression but also may lead to more aggressive disease [68]. Tumors overexpressing H-Ras (either due to H-ras mutations or upstream growth factor receptor signaling) showed increased expression of Notch 1, indicating that Notch may also be a downstream effector of oncogenic Ras.

Inhibiting Notch signaling seemed to suppress Ras-induced tumorigenesis, supporting a link between these pathways and a rationale for targeting both [69,70].

Interactions between Notch and the Akt, TGFB, Wnt, and HIF pathways may also exist and as novel agents targeting these pathways become available, combination approaches with Notch inhibitors could be considered [69,71,72].

2.2.4. Notch and tumor-initiating cells

In breast cancers and other cancers, there is now increasing support for the theory that a subpopulation of cancer cells exist known as tumor-initiating cells (TIC) or cancer stem cells. These cells are not only capable of self-renewal and proliferation but have also been implicated in both treatment resistance and disease relapse [73–75]. A population of $CD24_{\sqcup}$/low/$CD44_{\sqcup}$ cells, believed to represent TICs, has been isolated from breast cancers and are 1000 times more tumorigenic than cell populations lacking these cells, with injection of as few as 200 TICs causing tumor formation in SCID mice [72]. TICs, like normal stem cells, are dependent on a number of key signaling pathways including the Notch pathway. Using mammospheres (in which putative mammary stem cells are cultured in vitro within multicellular spheroids), Dontu et al. have shown that the self-renewal capacity of mammospheres is enhanced 10-fold when cultured in the presence of a synthetic peptide derived from the DSL (delta-Serrate-Lag2) domain, which is highly conserved in all Notch ligands and capable of Notch receptor activation. Conversely, mammosphere self-renewal was inhibited by Notch 4 blocking antibody or an inhibitor of the γ-secretase enzyme [76]. Similar findings have been reported by Farnie et al. who have shown that the efficiency of ductal carcinoma in situ derived mammosphere production was significantly reduced when Notch signaling was inhibited [77]. In primary breast cancer and breast cancer cell line-derived tumorspheres, Notch 3 and Jag1 have emerged as key regulators of TIC renewal and hypoxia survival [78,79]. Taken together, it would seem that targeting the Notch pathway might be one strategy to specifically target TICs, which may be more resistant to conventional anticancer treatments.

2.2.5. Notch and triple-negative breast cancer

Triple-negative (TN) breast cancers represent about 20% of all breast cancers, and despite initial response to systemic treatment, this disease follows an aggressive course. Cell line data show that basal/TN cancers have elevated Jag 1 levels, and BRCA-1 mutant breast cancers, which are typically of the basal/TN subtype, show elevated Jag1 expression compared with their BRCA2 (predominantly luminal) counterparts [80]. Resection specimens from TN breast cancers show a statistically significant association between elevated expression of Notch ligands/receptors and the basal/TN subtype [54,56,81]. In a disease with a paucity of treatments, targeting the Notch pathway is currently under investigation. TN breast cancer is still therapeutically challenging, at least partly due to lack of molecular targets. Many ongoing studies are investigating the potential of targeting Rb/p16 pathway abnormalities and p53 mutations, which are associated with poor prognosis [82,83].

2.3. Wnt signaling

Wnt proteins are a family of secreted peptides with pivotal roles in various processes, including embryonic induction, generation of cell polarity, and cell fate specification [84]. Aberrant activation of Wnt signaling has been described in numerous solid cancers, including colorectal, ovarian and breast cancer [83–88]. Clinical and experimental evidence suggests that β-catenin expression associates with poor prognosis in breast cancer [89,90] and is enriched in basal-like breast cancer [87]. Loss of negative regulators of Wnt/β-catenin pathway, such as the extracellular inhibitor of Wnt secreted Frizzled-related protein 1 (*sFRP1*), is found in many breast tumors and is associated with poor prognosis [84,91]. Furthermore, the down regulation of Wnt inhibitor Dickkopf 1 (*DKK1*) in a lung metastasis-derived cell line (MCF7-LM) also suggests the importance of Wnt regulation in the metastatic process in breast cancer [92]. Altogether, these data strongly suggest that Wnt pathway deregulation in the breast microenvironment contributes to cancer formation and metastasis (Fig. 2).

Emerging evidence suggests that breast cancer initiation and maintenance may be regulated by a small population of cells within the tumor, either stem cells or cells that exhibit stem-like properties [93]. Transplantation experiments performed in immunocompromised mice showed that only approximately 100 human breast cancer cells expressing cell surface markers $CD44^{+}CD24^{-/low}$ were tumorigenic and could be serially passaged to generate new tumors [94].

Cells isolated from human breast cancers marked by $CD44^{+}CD24^{-/low}$ lineage are anoikis-resistant and capable of self-renewal as mammosphere (MS) colonies providing a link between MS and cell surface markers that enrich for tumorigenic cells [95,96].

The expression of Wnt1 in human mammary epithelial cells increased stem cell self renewal, resistance to apoptosis and failure to senescence [97]. A more recent study using the MMTV-WNT-1 mouse model has identified an expanded mammary stem cell (SC) pool from a population of committed luminal progenitors, indicating that Wnt-1 activation may induce the appearance of aberrant progenitor cells, and pointing out both mammary stem and progenitor cells as the cellular targets of Wnt-1-induced tumorigenesis [98].

Moreover, Wnt pathway activation increases radio resistance of progenitor cells in the mouse mammary gland and human breast cancer cell lines [99,100], indicating that Wnt signaling pathway is involved in resistance to current anticancer drugs, potentially by regulation of stem and progenitor cell populations.

The expression of Wnt pathway target genes correlates with the expression of estrogen receptor (ER, and molecular sub-type, and some genes predict prognosis. Wnt signaling would be more activate in breast cancer stem-like cells compared to normal stem-like cells. It was reported that Wnt pathway inhibition preferentially reduces stem-like cell activity in patient-derived metastatic breast cancer compared to normal cells [101], suggesting the potential of Wnt targeting therapeutics in breast cancer.

2.4. SHH signaling

The Sonic Hedgehog (SHH) signaling pathway plays a critical role in organizing cell growth and differentiation during embryonic tissue patterning [102–106], and is important in mouse mammary gland development. Disruption of its downstream transcriptional targets, such as the Patched homolog-1 (PTCH-1) or glioma-associated oncogene-2 (GLI-2) genes results in severe defects in ductal morphogenesis, such as human breast ductal dysplasia [107,108]. An in vitro study showed that disruptions of these genes also occur in breast cancer [108], suggesting a role for the SHH pathway in breast tumorigenesis. It was hypothesized that SHH activation contributes to the relapse of breast cancer, based on the observation that SHH mRNA, PTCH-1 mRNA, GLI-1 mRNA, and SMOH mRNA higher expression in breast tumors correlates with malignant cell invasiveness, and may be a predictor of postoperative relapse [109].

2.5. BRK pathway

Breast tumor kinase (BRK) is a non-receptor tyrosine kinase first identified while screening for protein tyrosine kinases in cultured human melanocytes [110], and later in breast tumors [111]. BRK is overexpressed in more than 60% of breast cancer patients, but not in normal mammary glands or benign lesions [112,114]. BRK overexpression was also reported in other cancers, including metastatic melanoma [115], colon cancer [116], squamous cell carcinoma [117], prostate

cancer [118], lymphoma [119], high-grade serous carcinoma, and ovarian cancer [120].

BRK is an encoded 451 amino acid polypeptide composed of a Src homology 3 (SH3) domain, a SH2 domain, and a kinase domain displaying 30–40% sequence identity with Src kinases [121]. However, unlike Src kinases, BRK lacks the myristoylated N-terminal consensus sequence required for membrane anchorage, explaining its localization in the nucleus and the cytoplasm. Nonetheless, BRK is regulated negatively by phosphorylation of the C-terminal tyrosine 447 (analogous to the regulatory Y530 of Src) and positively by phosphorylation of tyrosine 342 in the catalytic domain (analogous to Y419 of Src) like SRC kinases [122,123]. Others and Lukong's research group have shown that mutation of tyrosine 447 to phenylalanine significantly enhances the kinase activity of BRK [122,123].

The cellular roles of BRK in breast cancer have not been fully elucidated. However, experimental evidence suggests that this tyrosine kinase may be a major player in breast tumorigenesis. For instance, the overexpression and constitutive activation of BRK in non-transformed human mammary epithelial cells or BRK-negative breast cancer cells induced anchorage-independent growth and increased cell survival, respectively [125,126]. BRK enhances EGFR tyrosine kinase signaling and positively regulates breast cancer cell growth and migration [125–131]. The expression of BRK was highest in cancers that also expressed HER2 and HER4 [113,132]. Although no specific BRK signaling pathway has been delineated, BRK is implicated in several signaling cascades. Notably, consistent with its potential roles in breast tumorigenesis, BRK associates with EGFR, which enchances mitogenic signals by promoting the metabolic signaling pathway phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt). BRK-EGFR interactions also stimulate cell migration by activating signaling molecules such as mitogen-activated protein kinase (MAPK) and paxillin [125–128,133]. In addition, BRK promotion increased cell survival, delayed involution, and latent tumor formation by inducing p38-driven pro-survival signaling pathways, in a mouse model [134].

On the same hand, depletion of BRK in breast cancer cells impaired the activation of EGFR-regulated signaling molecules [135]. Significantly increased MAPK activity, cell proliferation and migration were shown in breast cancer cells stably expressing BRK-Y447F, and conversely, a decreased migration was observed in breast cancer cells depleted of BRK [136]. Altogether, these findings strongly suggest a role for BRK in promoting cell proliferation and migration.

Groundbreaking findings from studies aimed at identifying and characterizing of BRK-interacting proteins and substrates have significantly improved our understanding of the molecular and cellular functions of BRK. Lukong's research group showed that the BRK substrate Sam68 (Src associated during mitosis, 68 kDa) is an effector of EGF stimulation and that BRK contributes to Sam68 phosphorylation in the EGF-treated breast cancer cells [124,137]. Other substrates such as paxillin [128], serine/threonine kinase protein kinase B/Akt [23], insulin receptor substrate-4 (IRS-4) [138], signal transducer and activator of transcription 3 (STAT3) [139], STAT5b [140], p190 [128,133], kinesin-associated protein 3A [141] and polypyrimidine tract-binding (PTB) protein-associated splicing factor (PSF) [142] also link BRK to signal transduction. For example, STAT3 phosphorylation (activation) by BRK, resulted in increased cell proliferation [139]. However, the STAT3 target gene product suppressor of cytokine signaling 3 (SOCS3) may inhibit BRK-induced activation of STAT3 [143], indicating that BRK interactions with STAT3 signaling are complex. In addition, BRK phosphorylation of paxillin and p190 results in the activation of the small GTPase Rac1 and the induction of cell migration and cell invasion in an EGF-dependent manner [128,133]. BRK was also shown to phosphorylate STAT5b on Y699, enhancing STAT5b transcriptional activity, which in turn promotes the proliferation of breast cancer cells [140]. Interestingly, Lukong's research group demonstrated that KAP3A is required for BRK-induced cell migration and that the phosphorylation of the protein-associated splicing factor (PSF) by BRK results in cell cycle arrest [141,142]. Furthermore, stimulation of insulin-like growth factor-1 receptor (IGF-1R) in human breast cancer results in the activation of BRK [138]. Heat shock protein 90 (Hsp90) was recently identified as a BRK interacting protein and shown to stabilize BRK in breast cancer cells [144]. BRK has been shown to regulate clathrin-mediated EGFR endocytosis via phosphorylation of ARAP1 (Arf-GAP, Rho-GAP, ankyrin repeat, and pleckstrin homology (PH) domain-containing protein 1) [145] and also to interact with EGFR and inhibit ligand-induced EGFR degradation [146].

Notably, the overexpression of BRK results in the phosphorylation of numerous cellular targets still to be unraveled [135,142]. In a recent proteomic study, downstream of tyrosine kinase 1 (Dok1), a tumor suppressor, was identified as a potential substrate of BRK [147]. To further understand the cellular roles of BRK, Lukong's research group explored the functional link between BRK and Dok1. Dok1 is a scaffolding protein which mediates protein–protein interactions and has been shown to be phosphorylated by several tyrosine kinases including SRMS, v-Src, c-Abl and p210-Bcr-Abl [148–153]. These authors showed that BRK phosphorylates Dok1 predominantly on Y362, promoting its proteasome-mediated degradation [154].

2.6. HER signaling

Human epidermal growth factor receptors 1–4 constitute a family of tyrosine kinase receptors expressed in normal tissues and in many types of cancer. Human epidermal growth factor receptor-2 (HER2/neu, c-erbB2) is amplified in various human breast cancer cell lines [155]. HER2 signaling amplification results in Her2 protein overexpression which is linked to tumor cell proliferation and cancer progression [156]. Targeted therapies are developed to bind specific molecules in signaling pathways important for cancer development and progression, providing most effective therapy in appropriately selected patients.

HER2 testing is used to select patients for potentially resistant and expensive therapy. The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) identified criteria and areas requiring clarification to improve the accuracy of HER2 testing by immunohistochemistry (IHC) or in situ hybridization (ISH)[157].

HER2 extracellular domain (ECD) levels can be found elevated in patients with HER2 negative tumors (by IHC or FISH/CISH/SISH) [158,159].

Immunohistochemical detection (IHC) uses monoclonal or polyclonal antibodies that bind to the protein. Currently in the U.S.A. there are two test approved for HER2 assessment: HerceptTestTM (DAKO, Glostrup, Denmark) and HER2/neu (4B5) rabbit monoclonal primary antibody (Ventana, Tucson, Arizona). HER2 testing results by IHC allows grouping of patients in three categories (positive, equivocal and negative) requiring different management strategies [160].

HER2 molecular analysis has become an integral part of the diagnostic breast cancer patient work-up. The principles of in situ hybridization are simple: use of labeled DNA probes complementary to genomic sequences of interest and hybridization of them to the target tissue.

Fluorescent in situ hybridization (FISH) utilizes fluorescence microscopy and positive result is defined as an average of more than 6 HER2 gene copies per nucleus for test systems without an internal control probe or HER2/CEP17 ratio of more than 2.2 where CEP17 is a centromeric probe for chromosome 17 on which the HER2 gene resides. The negative result is defined as less than four copies of HER2 gene per nucleus for systems without a probe, or HER2/CEP17 ratio of less than 1.8. The equivocal range for HER2 FISH assays is defined as HER2/CEP17 ratios from 1.8 to 2.2 or average gene copy number between 4.0 and 6.0 [158,161].

Chromogenic in situ hybridization (CISH) uses chromogens for signal identification with several advantages over FISH: permanent staining, use of bright field microscopy, easy identification of the target cells [162].

Silver enhanced in situ hybridization (SISH) is a highly sensitive technique with permanent staining, thus allowing specimen archiving [163].

HERmark® test is a new assay that uses the VeraTag system to identify total levels of a cellular protein or two similar or dissimilar proteins in close proximity. This method allows the measuring of total HER2 protein and the amount of HER2 homodimers in breast cancer tissue. According to study results in metastatic HER2 positive breast cancer patients treated with trastuzumab, the assay was superior to FISH and to immunohistochemistry in predicting benefit from trastuzumab therapy [164]. In a preliminary report from recently completed adjuvant trial, the HERmark assay declassified 25% of cases originally classified as positive, equivocal, or negative when done by central IHC [165].

2.7. Others pathways

Normal mammary stem cells (MSC) are responsible for the generation of adult mammary tissue and the distinct cell types within it as well as the extensive remodeling and enlargement of the gland during multiple cycles of pregnancy [166,167]. Mammary development is controlled by a variety of hormones, including estrogen without which development cannot occur [168]. Isolation of MSC using cell sorting techniques has allowed extensive studies of this cell sub-population and it has been shown that these cells lack estrogen receptor alpha (ER) [169,170]. In order to respond to systemic hormone signaling, these cells must, therefore, rely on local mediation of the signals by ER positive cells. There is good evidence that the epidermal growth factor receptor (EGFR) pathway, via binding of the amphiregulin ligand, is responsible for paracrine signaling that induces epithelial proliferation during ductal elongation of the mammary tree, but it is unknown whether this signal affects stem cells [171].

The development and progression of breast tumors has been proposed to be driven by breast cancer stem cells (CSC) identified by the cell surface phenotype $ESA^{+}CD44^{+}CD24^{low}$ or aldehyde dehydrogenase (ALDH1) activity [172,173]. CSCs generate tumor heterogeneity and are able to reinitiate tumors in transplantation experiments [172]. CSCs are thought to be responsible for tumor recurrence as they have been shown to be inherently resistant to therapies, such as chemotherapy [174], radiotherapy [175] and endocrine treatment [176,177].

There have been conflicting reports about the effects of estrogen on breast CSCs with evidence reported that estrogen can increase or decrease CSC number in breast cancer cell lines [178,179]. We predicted that these very different effects were due to the duration of hormone deprivation [177] compared to growth in standard conditions. In the current study, where estrogen treatment is initiated after hormone withdrawal, estrogen increases CSC activity and frequency measured both in vitro and in vivo. This is likely to be through paracrine regulation since breast CSCs are mainly ER negative. Here we establish that the EGF and Notch receptor signaling pathways are strong candidates as paracrine mediators of estrogen effects on CSC activity.

The PI3K/Akt/mTOR pathway also plays an important role in modulating responses to estrogen receptor (ER) therapy in a ligand-independent fashion, with several studies indicating that hyper-activation of Akt, and the subsequent hyper-activation of downstream mTOR, underlies resistance to endocrine therapies [180–182]. In breast cancer patients, activation of Akt is associated with a worse outcome among patients receiving endocrine therapy, with reduced clinical benefit in patients with positive expression of activated Akt [183]. Other studies have also shown an inverse correlation between Akt activation and partial response (PR) rates [184]. Expression of phosphorylated S6 kinase, a downstream marker of mTOR activation, significantly predicts overall survival in patients with hormone receptor-positive breast cancer receiving adjuvant endocrine therapy [185]. This is mimicked in vitro and in xenograft studies, where breast cancer cells with constitutive Akt activation exhibit reduced estrogen dependency and also demonstrate reduced sensitivity to anti-estrogen therapy [186]. In these studies, mTOR inhibition restored anti-estrogen sensitivity [185].

3. Signaling pathways and hormones inhibitors in breast cancer

3.1. CDK inhibitors in breast cancer

Breast cancer is the most common cancer in women worldwide [186] and some alterations of the cell cycle have been detected in this disease. Checkpoint deregulations play a key role in some breast cancers. Alterations of pathways that include cyclin, CDK, endogenous CDKI and Rb protein are seen in nearly all cancers, including breast cancer. Cyclin D1 and cyclin E overexpression, decreased expression of CDKI p27Kip1 are some of them in human breast cancer [187,188]. Cyclin D1 amplification is seen in nearly 60% of breast cancers. Estrogen uses cyclin D1 as one of its target genes to mediate its mitogenic effects. Some studies suggested that among patients with high tumor expression of cyclin D1, overexpression of HER2 was associated with reduced recurrence-free survival and tamoxifen responsiveness [189]. Overexpression of cyclin D1 changes the antagonistic effect of tamoxifen to an agonistic effect. Therefore tamoxifen resistance might be predicted with cyclin D1 overexpression [190]. However, this data has not been exactly verified and the prognostic significance of cyclin D1 overexpression is not completely understood.

There are a lot of CDKIs that have gone through or are currently tested in ongoing clinical trials in cancer treatment [191–194]. Most of them are targeting multiple CDKs, but some are targeting specific CDKs. Selective inhibition of CDKs is much better than non-selective, because more adverse and toxic effects have been seen with non-selective inhibitors. For instance, palbocyclib, a selective CDKI, exerts its killing effect on tumor cells rather than on normal cells. Various types of cancers including leukemia, melanoma, liposarcoma, hepatocellular carcinoma and breast cancer are being tested for palbocyclib effectiveness [195].

Understanding the biology of a tumor is a prerequisite for selecting an appropriate treatment. It is well known that CDK4/6 binds cyclin D1 for phosphorylation of Rb protein and activation of E2F transcription factors to progress cell cycle. If this well known pathway is blocked somewhere, the cell cycle progression will be arrested [196]. However, this mechanism is not adequate for someone to hypothesize that cyclin D1-overexpressing tumors will respond to any blockage in this pathway. For instance, mantle cell lymphoma, which is a high grade tumor, overexpresses cyclin D1 in 90%, yet CDK4/6 inhibitor achieves only 18% response rate. Mantle cell lymphoma cells may be dependent on cyclin D1 for their proliferation but not for their survival or any other resistance mechanisms may occur [197]. In particular, studies in HER2-induced mice mammary cancer models suggest that CDK4 and cyclin D1 are required to grow and to maintain tumor cells. In low-grade and cyclin D1-overexpressing malignancies, such as ER positive breast cancer, CDK4/6 inhibitor may have therapeutic potential. Cytoxic agents or targeted agents prevent tumor enlargement rather than tumor shrinkage. Palbocyclib, an oral CDK4/6 inhibitor breaks the above-described pathway, blocks Rb phosphorylation and subsequently induces G0/G1 arrest in sensitive cell lines.

The efficacy of palbocyclib was first tried in mouse models. Palbocyclib alone was found to be active and inhibited cell progression in in vitro studies [198]. ER-positive cell lines, including those with HER2 amplification, were most sensitive to growth inhibition by palbocyclib while nonluminal/basal subtypes were most resistant. Analysis of variance in both sensitive and resistant cells suggested that Rb protein and cyclin D1 were elevated and CDKN2A, which encodes p16, an endogenous inhibitor of CDK4 and CDK6, was decreased in most sensitive lines. Cell cycle analysis showed G0/G1 arrest in sensitive cell lines [24]. We know that tamoxifen and transtuzumab are more efficacious in ER-positive and HER2-amplified breast cancers, respectively. In an in vitro study that Finn et al. conducted, they identified a subgroup of patients most likely to benefit from palbocyclib: the

ER-positive luminal subtype [198]. They also identified potential synergy with standard therapies, like tamoxifen and transtuzumab. Another result of this study was that elevated cyclin D1 and Rb expressions and decreased p16 expressions in tumor tissue were indicators of response of palbocyclib.

To understand the efficacy of palbocyclib Dean et al. conducted a study in which surgically resected breast tumors were cultured with or without palbocyclib [199]. Regardless of ER or HER2 status, only Rb-positive tumor cell showed growth inhibition in response to palbocyclib. Tumors lacking Rb were completely resistant. This result characterizes Rb as the predominant target of CDK4/6 and the primary marker of drug response in breast cancer cells. This study also suggested the importance of direct screening of tumors for RB expression to select patients appropriate for palbocyclib treatment.

In order to understand whether combination therapy of palbocyclib with any chemotherapeutic agent is effective, palbocyclib was used with carboplatin [197]. Although carboplatin is an agent not used for first-line treatment in breast cancer, it is used in the metastatic setting. Palbocyclib 150 mg/kg/day was combined with carboplatin in mouse models with metastatic mammary cancer and this combination achieved statistically better results than carboplatin alone; however, palbocyclib was not found to be efficacious in Rb-deficient mice. In addition, no extramyelo suppression with the combination of chemotherapy and palbocyclib vs chemotherapy alone was observed.

Another study [200] was carried out to investigate the efficacy of palbocyclib in combination with doxorubicin in triple-negative breast cancer cell lines. Again, Rb expression was of paramount importance in determining response to either monotherapy with palbocyclib or combination treatment. In Rb-deficient cancer cells, CDK4/6 inhibition had no antitumor effect. Although in Rb-expressing cancer cells palbocyclib and doxorubicin provided a synergistic cytotoxic effect, doxorubicin-induced cytotoxicity was substantially reduced when combined with palbocyclib.

Palbocyclib was tried in combination with letrozole in a phase 1 study to assess tolerability and safety [201]. The combination was well tolerated and it was safe in 12 postmenopausal patients with ER-positive, HER2-negative breast cancer patients. Antitumor activity was seen in this trial. In combination with letrozole 2.5 mg per day, the recommended dose of palbocyclib was determined as 125 mg per day for 3 weeks followed by 1 week off (schedule 3/1). Based on this phase 1 trial palbocyclib was designed for a phase 2 clinical trial [201].

The combination of palbocyclib and letrozole was compared against letrozole alone in a phase 2 study in165 patients with advanced breast cancer. The study consisted of two parts: part 1 enrolled patients with ER-positive and HER-2 negative disease without other selection criteria; part 2 enrolled postmenopausal ER-positive, HER2-negative patients with cyclin D1 amplification and/or loss of p16 by fluorescent in situ hybridization. The addition of palbocyclib to letrozole prolonged median time to disease progression to 26.1 months compared with 7.5 months for letrozole alone. Palbocyclib and letrozole combination provided surprising improvement in progression free survival in this population. According to measurable response, 45% receiving the combination had measurable response, while in the letrozole group only 31% had measurable response. After 6 months follow-up period tumor shrinkage and/or stable disease rates were 70% in the combination group and 44% in the letrozole alone group. Eventually, palbocyclib prolonged median progression free survival by over 18 months [202].

3.2. Therapeutic implications of Notch inhibitors

There is now significant interest in developing therapies targeting the Notch pathway in breast cancer. A number of genetic and pharmacologic approaches are either available or theoretically possible to block Notch signaling at different levels of the cascade. Notch receptors and ligands may be inhibited by selective strategies including monoclonal antibodies, antisense, or RNA interference; nonselective strategies including soluble ligands, receptor decoys; or inhibition of enzymes involved in glycosylation or cleavage of receptors, such as γ-secretase inhibitors (GSIs) or ADAM inhibitors, are also being explored.

At the present time, the GSIs originally developed as potential inhibitors of the presenilin g-secretase complex that cleaves B-amyloid peptide (which leads to Alzheimer's disease through plaque formation) are the furthest in development as potential anticancer agents [207, 208]. GSIs show antitumor activity in several human cancer cell lines. Xenograft studies with glioblastoma and lung adenocarcinoma cell lines have shown that GSIs reduced both tumor growth and vasculature, induced growth arrest of T-ALL cells, and induced apoptosis in melanoma cell lines [203]. They have also been shown to effectively induce apoptosis in TN MDA-MB-231 cells. In ER␣ MCF7 cells, enhanced killing was seen when GSIs were combined with the anti-estrogen tamoxifen, suggesting that antiestrogen treatment in ER-cells may activate Notch signaling, which is then blocked by concurrent treatment with a GSI inhibitor [204]. GSI treatment of numb-deficient in vitro-cultured tumor explants resulted in both, decreased cell proliferation (as measured by Ki67) and decreased expression of the glucose transporter Glut1, suggesting positron emission tomographic (PET) imaging could be one modality used to measure response to GSI treatment [205,206]. Preclinical studies in MDA-MD-231 breast cancer cells have also shown that GSI when combined with ionizing radiation may have additive effects [207]. Like other small-molecule inhibitors, GSIs have multiple downstream effects by targeting all Notch receptors, some ligands, ErbB4, syndecan, CD44, and other proteins. As a result, determining Notch pathway activity alone may not be the best predictor of response and it will be critical to develop biomarkers that accurately predict sensitivity to the GSIs. Mechanism-based toxicities will also have to be addressed by a careful choice of therapeutic agents, combinations, and regimens [208,209].

In a phase 1 trial of the GSI MK-0752 (Merck), given to 7 patients with advanced solid tumors and 14 patients with advanced breast cancer, the main adverse effects of continuous dosing included diarrhea, constipation, nausea, and abdominal cramping. Intermittent dosing schedules are now being investigated. Importantly, MK-0752 at all doses inhibited γ-secretase with a decrease in plasma Abeta40 (product of γ-secretase cleavage) by 46% at 4 h on day 1 compared with predose levels [210]. There are several ongoing clinical studies involving MK-0752 in breast cancer including 1 study exploring different dosing schedules, a study of MK-0752 in combination with tamoxifen or letrozole to treat early-stage breast cancer, and a phase I/II study of MK-0752 followed by docetaxel in advanced or metastatic breast cancer [211–213].

Another GSI, RO4929097 (Roche), shown in Fig. 3, has also entered clinical trials. This drug has been evaluated in 2 dosing regimens—days 1–3, 8–10 every 3 weeks, and days 1–7 every 3 weeks, and found to be generally well tolerated, with the most common adverse events being reversible fatigue, nausea, emesis, diarrhea, hypophosphatemia, and rash. In this study, pretreatment IL-6 and IL-8 levels have emerged as possible response predictive markers and will be explored further [214]. There is currently a phase II study in advanced TN breast cancer in which baseline and 4 to 5 week post-treatment tumor biopsies will be collected to explore the effect of the drug on components of the Notch pathway and surrogate markers of Notch inhibition. Drug exposure will also be explored through paired blood sampling. Another study combines R04929097 with the hedgehog inhibitor GDC-0449 (Genentech) in advanced breast cancer [215]. This combination is based on the theory that both the notch pathway and the hedgehog signaling pathway play important roles in self-renewal pathways and also interact with one another [216]. A potential challenge of targeting self-renewal pathways is clearly to ensure that normal cellular pathways dependent on self-renewal remain unaffected [217].

3.3. Inhibitors of the PI3K/Akt/mTOR pathway

3.3.1. mTOR inhibitors — the rapalogs

Rapamycin, a macrolide antibiotic, originates from *Streptomyces hygroscopicus* found in the soil on the island of Rapa Nui. Rapamycin (and its analogs, also known as rapalogs) acts by binding to the FKBP12 binding protein, which in turn interacts with the mTORC1 complex, inhibiting downstream signaling [218]. Rapamycin, the first defined mTOR inhibitor, specifically inhibits mTOR, resulting in inhibition of cell growth, cell cycle progression and cell proliferation [219]. However, the poor aqueous solubility and chemical stability of rapamycin restricts its application for cancer therapy [220]. The other rapalogs, synthetic derivatives of rapamycin with improved pharmaceutical properties, are temsirolimus, everolimus and ridaforolimus (formerly known as deforolimus) [221].

Though the rapalogs trace their history back to use as immunosuppressant drugs used in transplant medicine, their antiproliferative effects led to investigation of their use as anti-cancer agents [222,223]. As the first-generation mTOR inhibitors, rapamycin and its analogs (rapalogs) have proven effective in a range of preclinical models. In the clinic, rapalogs have demonstrated important clinical benefits, particularly against endometrial cancer, mantle cell lymphoma and renal cancer. Nevertheless, the overall objective response rates in major solid tumors achieved with single-agent rapalog therapy have been modest [224–226].

3.3.1.1. The rapalogs in combination chemotherapy. The rapalogs have been investigated as monotherapy in a host of other phase II studies in diverse tumor types, including neuroendocrine tumors, breast cancer, endometrial cancer and sarcomas [227]. Encouraging single agent clinical efficacy was observed with the use of everolimus in pretreated patients with recurrent endometrial cancer, where loss of PTEN expression was predictive of clinical benefit [228].

Despite the high expectation for their application in oncology based on sound rationale related to the presumed mechanism-of-action, the rapalogs monotherapy has only met with modest success. Most notable is their utility of these agents in combination therapy in breast cancer. The rapalogs in combination with other chemotherapeutics have shown early encouraging data. PI3K pathway activation has been found to lead to resistance to trastuzumab in HER2-overexpressing breast cancer [229]. In this regards, studies have investigated combining everolimus with trastuzumab and paclitaxel in women with prior resistance to the latter two agents. Confirmed partial responses were seen in 20% of subjects and stable disease in a further 56% in a phase II study [230].

The same strategy has been evaluated in a phase I trial of everolimus, trastuzumab and vinorelbine, achieving a disease control rate of 80% (37 of 46 evaluable patients) [231]. Recent data from two phase I trials suggest that everolimus can help overcome resistance to trastuzumab in women with HER-2+ MBC. Everolimus plus trastuzumab and weekly paclitaxel was shown to slow tumor growth in 77% of patients, and the combination of everolimus with trastuzumab and vinorelbine halted tumor growth in 62% of patients [232,233]. Although early indications suggest that targeting components of the PI3K pathway may have some activity in the treatment of metastatic breast cancer (MBC), additional data, including an understanding of combinations and patient selection, are required. However, in unselected patients with breast cancer these agents have modest anti-tumor activity in the range of around 10% [234].

mTOR targeting therapeutics such as everolimus and temsirolimus have not only been tested in combination with ErbB2 inhibitors but also with endocrine therapy. A phase III trial investigated letrozol with or without temsirolimus in ER positive, metastatic breast cancer [235]. The trial was terminated early because of increased toxicity and lack of efficacy. However, a more recent phase II trial assessing letrozol with or without everolimus in the neoadjuvant setting showed a marginally significant increase of the response rate in the combination arm [236]. More importantly yet, the phase III BOLERO-2 trial has investigated the combination of the aromatase inhibitor exemestane plus everolimus in patients with advanced breast cancer [237]. Taken together, the clinical results obtained with mTOR inhibitors are strongly dependent on the chosen concomitant therapy. This dependence on combination therapy is a feature often observed in targeted therapies.

3.3.1.2. Rapamycin-resistant mTOR function. Several recent studies highlight the emergence of rapamycin-resistant mTOR function in protein synthesis, cell growth, survival and metabolism [238. The limited effectiveness of rapamycin as cancer therapy can be explained first by its biochemical mechanism as well as its complex and variable signaling responses in cancer cells. Rapamycin in complex with the 12 kDa FK506 binding protein (FKBP12) partially inhibits mTOR through allosteric binding to mTORC1. This drug mechanism does not block all mTORC1 outputs and does not directly target mTORC2-dependent AKT function [238]. Generally, the activity of rapalogs in a host of tumor types where the PI3K/Akt/mTOR pathway is frequently activated has been disappointing. As a general rule, these agents only inhibit the mTORC1 complex (although there are some cellular models where disruption of mTORC2 also occurs [239]. Therefore, there have been legitimate concerns that their efficacy may be partly limited by a failure to stop mTORC2 mediated phosphorylation and activation of Akt.

The second factor contributing to rapamycin resistance is the mTORC1 negative feedback regulation of PI3K pathway. In preclinical and clinical settings, treatment of certain tumor types with rapamycin elevates PI3K–AKT activity and counteracts the therapeutic potential of mTORC1 inhibition, a phenomenon that is undesirable for cancer therapy [238]. This phenomenon can be explained as follow: inhibiting mTORC1 releases the feedback inhibition mediated by the S6KIRS1-PI3K loop that normally acts to moderate pathway activity. Thus this inhibition may lead to a paradoxical increase in Akt activity, a consequence of both biological and therapeutic implications. Indeed, increased phosphorylated Akt has been confirmed in tumor biopsies from rapalogs treated patients [240].

The third potential explanation for the limited activity of mTOR inhibitors in breast cancer and other tumor types may be related to a 'collateral effect' of mTOR blockade. mTOR inhibition blocks the natural negative feed-back on IGF-1R signaling exerted on PI3K [240]. This results in an increase in PI3K and Akt activations which could significantly counteract mTOR inhibition. Thus, dual inhibition of both IGF-1 signaling, with either MAbs against the receptor or tyrosine kinase inhibitors, and mTOR results in superior anti-proliferative effect over each single strategy. In the clinical setting, there is indirect evidence that this approach may be also beneficial. Octreotide was proven to inhibit IGF-1R signaling. Although octreotide has limited activity in patients with refractory neuroendocrine tumors, it has been shown to that the combination of everolimus and octreotide has resulted in an impressive activity [241].

It is therefore warranted to identify the subset of patients that may putatively benefit from it, and optimized PI3K/Akt/mTOR-dependency genetic signatures should be developed. In this direction, it has been recently observed that a majority of locally advanced and inflammatory breast cancers over-express the translation regulatory protein 4E-BP1 and the initiation factor eIF4G, both of them are mTOR downstream targets. While additional studies are planned to further dissect this interaction, it does seem reasonable to explore the benefits of mTOR inhibitors in the treatment of locally advanced breast cancer [242]. Taken together, these data suggest that pathway activation and reactivation could be avoided by PI3K, Akt or concomitant PI3K and mTOR catalytic inhibition (that would target both mTORC1 and mTORC2). Thus, a more complete suppression of mTOR global signaling network by the new inhibitors other than the rapalogs is expected to yield a deeper and broader anti-tumor response in the clinic [40].

3.3.2. Dual PI3K-mTOR inhibitors

These molecules simultaneously target the ATP binding sites of mTOR and PI3K with similar potency and cannot be used to selectively inhibit mTOR-specific activities [242–245]. Thus, they are generally not considered as useful research tools to study the mTOR regulation or function. However, they may have unique advantages over single-target inhibitors in certain disease settings because they can target at least three key enzymes (PI3K, Akt, and mTOR) in the PI3K signaling pathway. Inhibition of mTORC1 activity alone by rapalogs may result in the enhanced activation of the PI3K axis because of the mTOR-S6K-IRS1 negative feedback loop [240]. Therefore, the mTOR and PI3K dual specificity double targeting inhibitors might be sufficient to avoid PI3K pathway reactivation.

As mentioned previously, agents belonging to this class target all catalytic isoforms of PI3K, mTORC1 and mTORC2. This has the theoretical advantage of efficiently shutting down the PI3K/Akt/mTOR pathway but also the possible shortcoming of enhanced toxicity. SF1126 is a small molecule prodrug of LY294002 that is conjugated to an integrin-binding component. This design promotes delivery to the tumor together with its associated vasculature where cleavage leads to the active drug release. It has shown significant anti-tumor effects in xenograft models of solid tumors including glioblastoma, breast and prostate cancer, and potent anti-angiogenic activity has also been observed, partially related to a reduction in HIF-1α levels [246]. Two dual inhibitors are under investigation: Novartis (Basel, Switzerland) — NVP-BEZ235 and NVPBGT226. NVP-BEZ235 is a novel orally available product belonging to the class of imidazoquinolines [247]. NVP-BEZ235 binds the ATP-binding clefts of PI3K and mTOR kinase, thereby inhibiting their activities [83]. Preclinical studies demonstrated anti-proliferative activity against a wide range of cancer cell lines, including HER2-overexpressing breast cancer models of trastuzumab and lapatinib resistance [241,248]. Further, tumor growth suppression has been shown in PI3K mutated xenograft models of human cancer.

Increasing evidence showed that NVP-BEZ235 is able to effectively and specifically reverse the hyperactivation of the PI3K/mTOR pathway, resulting in potent antiproliferative and antitumor activities in a broad range of cancer cell lines and experimental tumor models [249,250]. In breast cancer cells, NVP-BEZ235 blocked the activation of the downstream effectors of mTORC1/2, including Akt, S6, and 4E-BP1 [241]. Especially, at doses higher than 500 nM, NVP-BEZ235 completely suppressed Akt phosphorylation, irrespective of exposure duration. 3.3. mTOR kinase inhibitors (selective mTORC 1/2 inhibitors).

Given the previous considerations with rapamycin, recently the discovery of small molecule ATP-competitive mTOR kinase inhibitors (TKIs) that bind to the active sites of mTORC1 and mTORC2, thereby targeting mTOR signaling function globally was reported [251–253]. These second-generation mTOR inhibitors bind to the ATP-binding site in the mTOR kinase catalytic domain (act as ATP-competitive inhibitors) and thereby indiscriminately inhibit both mTORC1 and mTORC2, downregulate mTOR signaling generally, and reduce the feedback activation of PI3K signaling in cancer cells. Therefore, they share more in common with the dual PI3K/mTOR inhibitors than the rapalogs in terms of their mechanism-of-action. In turn, this should mitigate the paradoxical PI3K activation consequent to de-repression of the negative feedback seen with rapalogs [251].

On the other hand, unlike PI3K/mTOR dual inhibitors, they selectively inhibit both mTORC1 and mTORC2 without inhibiting other kinases [251]. This class of agents includes PP242, PP30, Torin1, Ku-0063794, WAY-600, WYE-687 and WYE-354. Clearly, these mTOR kinase inhibitors have provided novel tools for elucidating new roles of mTOR in tumorigenesis. However, more studies are still required to understand the distinct effects and mechanisms between these pharmacological agents and rapamycin in targeting cancer cell growth and survival, and to evaluate their efficacy in the treatment of cancer and other diseases in which PI3K/Akt/mTOR pathway is hyperactivated [252].

Thus, the discovery of these specific, active-site mTOR inhibitors has opened a new era for breast cancer therapy. As discussed earlier, the development of highly potent and specific active-site inhibitors of mTOR not only provides invaluable tools for deciphering novel insights to the increasingly complex mTOR signaling network but also offers considerable new opportunities to fully exploit the therapeutic potential of mTOR targeting in cancer. Interesting preclinical data of two such agents (PP242 and PP30) suggests that they have more substantial anti-proliferative actions than rapamycin not because of the mTORC2 effects but rather because they are more effective in suppressing mTORC1 [251]. Other agents in this group include WAY-600, WYE-687, and WYE-354, the latter of which has displayed robust antitumor activity in PTEN null tumor xenografts [251]. AZD8055 (Astra Zeneca, London, UK), OSI-027 (OSI Pharmaceuticals, Melville, NY, US) and INK128 (Intellikine, La Jolla, CA, US) are the first mTOR kinase inhibitors to enter clinical trials [253].

3.3.3. Natural products

Increasing evidence has demonstrated that some natural products, including curcumin and resveratrol may be used as mTOR inhibitors. Recently it has been described that resveratrol activated AMPK in both ER-positive and ER-negative breast cancer cells, and consequently inhibited mTOR and its downstream 4E-BP1 signaling and mRNA translation [254]. Moreover, it was also suggested that curcumin may exert its anti-proliferative effects by inhibiting mTOR signaling and thus may represent a new class of mTOR inhibitors. Numerous studies have shown that curcumin inhibited the growth of a variety of cancer cells and showed effectiveness as a chemopreventive agent in animal models of carcinogenesis [255,256]. In numerous cancer cell lines, it has also been suggested that curcumin may execute its anti-cancer effect primarily through blocking mTOR mediated signaling pathways [255,257].

4. Therapeutic targeting of the microenvironment

New insights into the tumor microenvironment, both focused and global, are identifying novel therapeutic targets. Currently, three types of tumor microenvironment-targeting therapies are in clinical practice: aromatase inhibitors (which target the aromatase enzyme predominantly expressed by stromal components), angiogenesis-modulating agents (including anti-VEGF receptor antagonists), and inhibitors of HER family receptors (such as trastuzumab, which inhibits receptor signaling on epithelial cells triggered by stroma-produced growth factors).

Whereas aromatase inhibitors and trastuzumab have become standard therapy, the clinical effectiveness of angiogenesis inhibitors is less clear [258]. In addition, there is a concern that inhibition of angiogenesis may enhance disease progression based on data in animal models where treatment with anti-angiogenic agents increased invasiveness and metastatic spread [259,260]. Potential selection for hypoxia-tolerant clones or establishment of leaky, metastasis-promoting vessels could explain these results [261]. In addition to these concerns, bevacizumab, a clinically approved VEGF inhibitor, has been associated with significant adverse reactions, including hemorrhage, neutropenia, gastrointestinal perforation, and thrombo-embolic events. A recently published meta-analysis of 16 randomized controlled clinical trials administering bevacizumab demonstrated that this agent, when used in combination with chemotherapy, was associated with increased risk of fatal treatment-related adverse events compared to the use of chemotherapy alone [262]. Whereas targeting the tumor microenvironment is an exciting possibility, side effects resulting from disruption of homeostatic functions in normal tissues are very likely, as was demonstrated by the poor tolerability of MMP inhibitors. In the past years, numerous targets have been investigated in early clinical trials, including antibodies targeting FAP, c-Met antagonists and multi-targeted receptor tyrosine kinase inhibitors such as

sunitinib [263,264]. Some of these have been plagued by poor side effect profiles whereas others have been well tolerated, but ineffective.

In addition to drugs being developed against novel targets, the anti-tumor effects of several older agents seem to be mediated through microenvironmental actions. For example, bisphosphonates (e.g., zoledronic acid), which are used for the treatment of osteoporosis and the management of bone metastasis, are now recognized to have activity outside of the skeleton, including direct anti-tumor effects on the malignant epithelium, and modulating angiogenesis and immune cell infiltration [265].

Osteoclasts are an important component of the normal bone microenvironment as well as bone metastases. Metastatic tumor cells secrete growth factors that activate osteoclasts, which degrade bone and release additional growth factors, triggering a paracrine cascade that promotes tumor growth and bone destruction. Denosumab is a monoclonal antibody that binds RANKL and inhibits osteoclast function. Recently, denosumab was compared to zoledronic acid in a phase III randomized clinical trial in breast cancer patients with bone metastases [266]. The results of this trial demonstrated that denosumab was well tolerated, and superior to zoledronic acid in delaying time to complications of bone metastases (that is, pathological fractures) but did not improve survival.

An important new hypothesis in targeting the tumor microenvironment is the induction of microenvironmental 'reprogramming'. Rolny and colleagues [267] recently published an intriguing discovery that overexpression of histidine-rich glycoprotein (HRG) in murine syngeneic tumor models induced 'normalization' of TAMs and blood vessel structure. Importantly, this was associated with decreased breast tumor growth and pulmonary metastasis, and increased sensitivity to chemotherapy. The authors demonstrated that the effects of HRG were dependent on the presence of TAMs and in particular TAM conversion from the 'M2' pro-tumor/pro-angiogenic phenotype to the 'M1' anti-tumor/pro-inflammatory phenotype. In addition, HRG expression was associated with vessel normalization, which was also dependent on TAM activity. This study has linked the phenotypic switching of TAMs by HRG with orchestration of vascular normalization. This effect of HRG on TAMs seems to be mediated through the down-regulation of placental growth factor, though the precise mechanism is unclear.

Coussens and colleagues have recently described that the ratio of macrophages to T cells predicts clinical outcome, with increased macrophage recruitment associated with worse outcome [268]. Interestingly, cytotoxic chemotherapy induces the recruitment of TAMs into invasive carcinomas by increasing the expression of CSF-1, a macrophage-recruiting cytokine. Inhibition of TAM recruitment by several approaches increased the efficacy of chemotherapy by decreasing tumor development and metastasis in a $CD8^+$ xsT cell-dependent manner. The authors postulate that chemotherapy increases TAM recruitment that subsequently modulates T cells, favoring the $CD4^+$ T-cell phenotype, which leads to inhibition of anti-tumor immunity. Inhibition of TAMs promotes $CD8^+$ T-cell recruitment and is associated with increased anti-tumor immunity. These data support the development of novel compounds that target TAMs and, in concert with cytotoxic chemotherapy, can encourage anti-tumor immunity [263].

Response to chemotherapy can be assessed by changes in tumor size and imaging characteristics as well as histo-pathological assessment. Tumor growth can progress, stabilize or regress in response to chemotherapy. In the case of a good tumor response characterized by tumor shrinkage, it is possible that the tumor microenvironment actively participates in the tissue remodeling. A simplistic model would be that classic cytotoxic therapies kill tumor cells, which then gives stromal components the opportunity to 'mop up' the necrotic debris. An alternative hypothesis is that the microenvironment, either as a direct effect of chemotherapy or in response to signals derived from the assaulted epithelium, acquires an altered phenotype that independently inhibits tumor growth. Identification of these microenvironmental changes that take place during tumor regression have not been intensively studied. Such studies may identify 'reprogramming' events that can be pharmacologically mimicked with novel, non-cytotoxic agents. Such manipulation of the microenvironment to promote an anti-tumor phenotype in stromal components represents a novel treatment strategy.

Metronomic therapy refers to the frequent or continuous administration of low doses of chemotherapy with the goal of eliciting an anti-tumor response while minimizing side effects. Interestingly, metronomic therapies have been implicated in inhibiting angiogenesis, promoting a beneficial immune response and tumor dormancy [269]. The mechanisms by which metronomic therapies influence these changes are largely unknown. One possible explanation is that these chronic therapies are re-modeling the epigenetic landscape of the tumor microenvironment. Just as the epigenetic changes identified in tumors possibly arise from chronic exposure to pro-tumorigenic signals derived from malignant epithelium, one could postulate a similar affect from chronic exposure to anti-neoplastic agents.

Epigenetic therapies, such as the histone deacetylase inhibitor suberoylanilide hydroxamic acid (also called vorinostat), are currently under clinical investigation for the treatment of breast cancer. While developed to target the malignant epithelium, their effect on the microenvironment may induce alterations that help orchestrate an anti-tumor response. Currently, there are no reports of the gene expression or epigenetic profiles of tumor samples obtained from patients treated with metronomic therapy or histone deacetylase inhibitors. These data will be valuable to our understanding of the microenvironmental changes induced by these therapies

Besides identifying new therapeutic targets, the microenvironment has also been implicated in chemotherapy resistance. Weaver and colleagues [270], working with three-dimensional cultures, demonstrated that sensitivity to chemotherapy could be influenced by cellular polarity, which is mediated in part by integrin expression and exposure to basement membrane. Hiscox and colleagues [271] demonstrated that resistance to fulvestrant, an anti-estrogen, promotes an invasive phenotype secondary to increased epithelial expression of c-MET, which is then activated by fibroblast-produced HGF. Loeffer and colleagues [272] generated an oral vaccine against FAP and studied its effect on the growth of multidrug-resistant breast cancer in murine xenografts. The vaccine decreased tumor collagen I, an ECM component previously implicated in chemotherapy resistance, and tumors from these animals had a significant improvement in chemotherapy uptake as well as decreased tumor growth resulting in increased survival. These data demonstrate that, in addition to promoting progression, the microenvironment can modulate sensitivity or resistance to chemotherapy.

5. Conclusions

The era of targeted therapeutics has brought rapid progress to breast cancer treatment. Targeted therapies increase cure rates in localized and prolong survival in metastasized breast cancer. The list of targets for drug treatment has dramatically increased with a deeper understanding of the molecular pathology of breast cancer.

There is growing evidence that cross talk between Notch, Wnt, HHS and key signaling pathways, as well as their role in regulating TICs, can promote tumorigenesis in breast cancer. There is preclinical and early clinical evidence that agents targeting the Notch, Wnt, SHH and key pathways, of which the γ-secretase inhibitors, Cyclopamine and Quercetin are most advanced, may be effective in this disease.

Novel treatment strategies are urgently needed, especially in the setting of metastatic disease where outcomes are still dismal. The breast cancer microenvironment is a complex mixture of cells, the proteins they secrete, and the ECM in which they reside. Alterations within the microenvironment are now recognized during key steps of tumor

progression, making them attractive candidates for therapeutic modulation. The relative genomic stability of stromal cells makes the development of chemoresistance to stromal-target therapy less likely. Furthermore, the epigenetic modifications that contribute to phenotypic alterations, while inheritable, are reversible, and there is mounting interest in 'normalizing' the altered stroma, thereby abrogating its tumor-supporting role.

One major obstacle facing stromal-targeted therapy is avoiding disruption of homeostatic function in normal tissues. Despite these challenges, our improved understanding of key aspects of tumor progression should lead to treatment strategies that can discriminate normal tissue from neoplasm.

How the tumor microenvironment changes during chemotherapy-induced tumor regression is still poorly understood. Insights into these changes may identify important pathways, which can be activated using noncytotoxic therapies. As the mainstay of aggressive forms of breast cancer will continue to rely heavily on cytotoxic therapies for the foreseeable future, agents without these characteristics will be particularly valuable in combination trials.

Translating our growing knowledge of microenvironmental influences on tumor progression into clinical practice is challenging. For example, targeting bone marrow-derived mesenchymal cells, which influence both primary tumor growth and the metastatic niche, prior to clinically evident metastatic disease makes intuitive sense. However, testing these potentially important agents in early clinical trials of recurrent or refractory disease may not yield significant improvements in such advanced disease. Thoughtful clinical trial design, including neoadjuvant therapy during which pre- and post-treatment tumor samples can be analyzed, will be vitally important in developing stromal-targeted therapy. Despite these challenges, taken together, the majority of data support the rationale for targeting the tumor microenvironment in the treatment of breast cancer. Further clinical trials will hopefully confirm the efficacy of some of those pathways inhibition either as a single agent or in combination with endocrine therapy, targeted therapies, chemotherapy, or possibly even radiation therapy as novel approaches, ultimately leading to improved patient outcomes overall.

6. Authorship contribution

All authors contributed to the design, preparation, editing, and final review of the manuscript.

7. Conflict-of-interest disclosure

Authors declare no competing financial interests.

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