

# The crossroads of breast cancer progression: insights into the modulation of major signaling pathways

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**Abstract:** Cancer is the disease with highest public health impact in developed countries. Particularly, breast cancer has the highest incidence in women worldwide and the fifth highest mortality in the globe, imposing a significant social and economic burden to society. The disease has a complex heterogeneous etiology, being associated with several risk factors that range from lifestyle to age and family history. Breast cancer is usually classified according to the site of tumor occurrence and gene expression profiling. Although mutations in a few key genes, such as *BRCA1* and *BRCA2*, are associated with high breast cancer risk, the large majority of breast cancer cases are related to mutated genes of low penetrance, which are frequently altered in the whole population. Therefore, understanding the molecular basis of breast cancer, including the several deregulated genes and related pathways linked to this pathology, is essential to ensure advances in early tumor detection and prevention. In this review, we outline key cellular pathways whose deregulation has been associated with breast cancer, leading to alterations in cell proliferation, apoptosis, and the delicate hormonal balance of breast tissue cells. Therefore, here we describe some potential breast cancer-related nodes and signaling concepts linked to the disease, which can be positively translated into novel therapeutic approaches and predictive biomarkers.

**Keywords:** breast cancer, estrogen receptor, PI3K, MAPK, JAK/STAT, Wnt, TGF $\beta$ , NF $\kappa$ B

## Introduction

Cancer is one of the main causes of morbidity and mortality, being the second most prominent cause of deaths worldwide, with about 14 million new cases and 8.2 million deaths in 2012,<sup>1,2</sup> representing one of the major issues in public health. According to the World Health Organization, breast cancer is the malignancy with the highest incidence among women, accounting for 25% of all female cancers diagnosed. Despite recent improvements in diagnosis and treatment, breast cancer remains the leading cause of cancer mortality among woman, representing 7% of all cancer deaths. The incidence and prevalence of breast cancer is increasing, especially in industrialized countries in North America, Western and Northern Europe, and Oceania.<sup>3</sup> From 2006 to 2010, breast cancer accounted for 19.8% of diagnosed cancers in the US.<sup>4</sup> For 2017, a total of 252,710 new cases and 40,610 deaths due to female breast cancer are estimated in the US alone.<sup>1</sup>

The development of the mammary gland encompasses different stages, such as embryonic, prepubertal, pubertal, pregnancy, and lactation. In each of these stages, such processes as epithelial proliferation during pregnancy and secretory alveolar differentiation during lactation take place.<sup>5</sup> It has been proposed that breast cancer

can develop through different mechanisms, but starts most frequently with imbalanced activity of pathways involved in patterning and morphogenesis of mammary gland developmental stages.

Several breast cancer classifications have been proposed, according to their invasive characteristics, occurrence, histology, or molecular profiling. Based on their site of occurrence, tumors can be classified as lobular (located at breast lobules) or ductal (at breast ducts). Carcinomas may also arise from invasive epithelial cells (medullary carcinoma), mucus-producing cells (mucinous carcinoma, also called colloid carcinoma), or a subtype of invasive ductal carcinoma (tubular carcinoma).<sup>6,7</sup> Traditionally, breast cancer has been classified by immunohistochemical detection of hallmark receptors associated with distinct cell functions. Cancers derived from luminal cells are the most common types of breast cancer, expressing hormone receptors for ER, PR, or the amplified HER2/ErbB2 receptor for EGF-like growth factors. HER2 is a transmembrane glycoprotein belonging to a family of receptors that regulate cell growth, proliferation, survival, differentiation, and angiogenesis. Activation of the receptor and its subsequent phosphorylation lead to several signaling pathways, such as those of PI3K, MAPK, and PKC, and also to their pathophysiological functions.<sup>7,8</sup> HER2-overexpressing tumors are considered to be more malignant, overexpressing other genes, such as *GRB7* and *PGAP3*, and may display representative *TP53* mutation. Each category is usually targeted by a specific drug therapy.

Tumors lacking expression of all three of these receptors (ER, PR, HER2) are referred to as triple-negative breast cancers, which most often derive from cells of basal origin.<sup>9</sup> Molecular gene expression profiling has also redefined breast cancer subtypes as luminal A, luminal B, HER2-rich, and basal-like, which roughly parallel the immunohistochemical categories.<sup>10–12</sup> In addition, a more recently discovered claudin-low class of breast cancers has been defined.<sup>13</sup>

Claudins are a family of tight junction proteins that are expressed exclusively in epithelial cells.<sup>6,7,9</sup> Claudin-low tumors, which lack or display low levels of E-cadherin and claudin 3, 4, and 7, are more heterogeneous than basal and luminal A subtypes, in addition to displaying more extensive lymphocytic infiltrates, larger tumors, and a high expression of mesenchymal markers as their main features. This tumor subtype is also associated with young age of onset and lower survival rates when compared with the luminal A subtypes.<sup>14,15</sup> A summary of histological and molecular classification of the different breast cancer subtypes is presented in Table 1.

**Table 1** Breast cancer subtype classifications, based on site of occurrence and/or biomarker status

| Classification based on site of occurrence   |  |              |            |
|--|--|--------------|------------|
| <b>In situ carcinoma</b>                     |  |              |            |
| Lobular                                      |  |              |            |
| Ductal                                       |  |              |            |
| <b>Invasive (infiltrating) carcinoma</b>     |  |              |            |
| Medullary                                    |  |              |            |
| Mucinous                                     |  |              |            |
| Ductal                                       |  |              |            |
| Lobular                                      |  |              |            |
| Tubular                                      |  |              |            |
| Classification based on molecular biomarkers |  |              |            |
| Subtype                                      | Biomarker status   | Prognostic   | Prevalence |
| Luminal A                                    | ER <sup>+</sup> /PR <sup>+</sup> /HER2 <sup>-</sup> /Ki67 <sup>-</sup>   | Good         | ~30%       |
| Luminal B                                    | ER <sup>+</sup> /PR <sup>+</sup> /HER2 <sup>-</sup> /Ki67 <sup>+</sup>   | Intermediate | ~20%       |
| Luminal B                                    | ER <sup>+</sup> /PR <sup>+</sup> /HER2 <sup>+</sup> /Ki67 <sup>+</sup>   | Poor         | ~10%       |
| HER2-enriched                                | ER <sup>-</sup> /PR <sup>-</sup> /HER2 <sup>+</sup>                      | Poor         | ~15%       |
| Triple-negative/basal                        | ER <sup>-</sup> /PR <sup>-</sup> /HER2 <sup>-</sup>                      | Poor         | ~10%       |
| Claudin-low                                  | ER <sup>-</sup> /PR <sup>-</sup> /HER2 <sup>-</sup> claudin <sup>-</sup> | Poor         | ~10%       |
| Normal-like                                  | ER <sup>+</sup> /PR <sup>+</sup> /HER2 <sup>-</sup> /Ki67 <sup>-</sup>   | Intermediate | ~5%        |

Understanding the development of breast cancer, applying appropriate (and often patient-specific) treatment, and making an adequate prognosis is frequently mixed by the diverse histological patterns and biological/morphological features of the disease, as well as its clinical behavior and molecular characteristics. Additionally, the onset and progression of the disease are often related to lifestyle, race,<sup>10</sup> geographical variation, age at menarche or menopause, and family history,<sup>16</sup> attributable to high penetrance susceptibility genes such as *BRCA1* and *BRCA2*.<sup>17,18</sup> As a result, a combination of these factors leads to deregulation of signaling pathways that modulate the normal function and development of the mammary gland. Therefore, unraveling the molecular network that is behind this disease should provide guidance toward early (and more accurate) diagnosis, as well as better targets for possible therapy intervention. Here, we describe the roles of major signaling pathways in the normal development of the mammary gland and in breast cancer progression. A summary of the most important molecular alterations associated with breast cancer along these pathways is presented in Table 2.

## Estrogen receptor The ER pathway

Estrogen comprises a set of steroid hormones involved in the development and maintenance of reproductive, cardiovascular, bone morphogenetic, and immune and central

**Table 2** Main molecular alterations in pathways associated with breast cancer and respective targeted therapies

| Gene/protein                     | Alteration                | References    | Therapeutic intervention                        | References       |
|----------------------------------|---------------------------|---------------|---|------------------|
| <b>Estrogen receptor pathway</b> |                           |               |   |                  |
| ER $\alpha$                      | Upregulation              | 75            | SERD, SERM                                      | 102, 103         |
| ER $\beta$                       | Blockage                  | 106           | NA  |                  |
| HER2                             | Upregulation              | 78            | NA  |                  |
| GATA3                            | Mutation                  | 82            | NA  |                  |
| COUP-TFI                         | Upregulation              | 85, 86        | NA  |                  |
| NGFIB                            | Upregulation              | 87            | NA  |                  |
| ROR $\alpha$                     | Downregulation            | 88, 89        | NA  |                  |
| ERR $\alpha$                     | Upregulation              | 92            | NA  |                  |
| ERR $\beta$                      | Upregulation              | 92            | NA  |                  |
| ERR $\gamma$                     | Downregulation            | 92            | NA  |                  |
| GPR30                            | Upregulation              | 107           | G1, G15   | 106              |
| <b>PI3K pathway</b>              |                           |               |   |                  |
| Akt                              | Mutation                  | 126           | NA  |                  |
| PTEN                             | Mutation                  | 82, 116       | NA  |                  |
| PDK1                             | Upregulation              | 126           | NA  |                  |
| mTOR                             | Upregulation              | 128, 129      | BEZ235, BKM120, BGT226, XL765, XLI47, SF1126    | 113              |
| PIK3CA                           | Mutation                  | 117, 121, 122 | LY294002, wortmannin                            | 113              |
| NOTCH1                           | Mutation                  | 124           | NA  |                  |
| <b>MAPK pathway</b>              |                           |               |   |                  |
| Ras                              | Mutation                  | 135           | NA  |                  |
| Raf                              | Mutation                  | 135           | Sorafenib                                       | 159, 160         |
| MAP3K1                           | Mutation                  | 82            | NA  |                  |
| ERK1/2                           | Downregulation            | 154           | NA  |                  |
| JNK1/2                           | Downregulation            | 154           | NA  |                  |
| p38                              | Downregulation            | 154           | NA  |                  |
| <b>JAK-STAT pathway</b>          |                           |               |   |                  |
| STAT3                            | Upregulation/<br>mutation | 186           | CJ1383, LL12, S31201, SF1066, siRNAs            | 202–204, 206–208 |
| STAT1                            | Downregulation            | 184           | NA  |                  |
| STAT5                            | Upregulation              | 190           | FLL32, IS3295                                   | 205              |
| STAT6                            | Downregulation            | 177           | NA  |                  |
| JAK2                             | Upregulation/<br>mutation | 181, 182      | Momelotinib, ruxolitinib                        | 194              |
| JAK1                             | Mutation                  | 181, 182      | Momelotinib, ruxolitinib, INCB047986, INCB39110 | 194              |
| JAK3                             | Mutation                  | 181, 182      | NA  |                  |
| <b>WNT pathway</b>               |                           |               |   |                  |
| Dvl                              | Upregulation              | 233, 234      | NA  |                  |
| GSK3                             | Downregulation            | 244           | NA  |                  |
| $\beta$ -Cat                     | Upregulation/<br>mutation | 224–226       | NA  |                  |
| Axin                             | Mutation                  | 224–226       | NA  |                  |

(Continued)

**Table 2** (Continued)

| Gene/protein                          | Alteration     | References | Therapeutic intervention   | References       |
|---------------------------------------|----------------|------------|----------------------------|------------------|
| WNT5A                                 | Downregulation | 238–239    | NA                         |                  |
| LRP5                                  | Mutation       | 240        | NA                         |                  |
| ASPP2                                 | Downregulation | 242        | NA                         |                  |
| DKK1                                  | Upregulation   | 246        | NA                         |                  |
| <b>TGF<math>\beta</math> pathway</b>  |                |            |                            |                  |
| TGF $\beta$                           | Upregulation   | 268, 269   | Fresolimumab, galunisertib | NCT01401062, 298 |
| Snail                                 | Upregulation   | 277        | NA                         |                  |
| SMAD4                                 | Upregulation   | 275        | SMAD4 RNAi                 | 300              |
| Twist                                 | Upregulation   | 278        | NA                         |                  |
| Mir200                                | Downregulation | 281        | NA                         |                  |
| Mir34                                 | Downregulation | 285        | NA                         |                  |
| <b>NF<math>\kappa</math>B pathway</b> |                |            |                            |                  |
| ERBB2                                 | Upregulation   | 338–340    | NA                         |                  |
| cRel                                  | Upregulation   | 335–338    | NA                         |                  |
| p50                                   | Upregulation   | 335–338    | NA                         |                  |
| p100                                  | Upregulation   | 351        | NA                         |                  |
| TP53                                  | Mutation       | 82         | Bortezomib                 | 358              |
| p52                                   | Upregulation   | 335–338    | NA                         |                  |
| I $\kappa$ B                          | Mutation       | 354        | NA                         |                  |
| p65                                   | Upregulation   | 335–338    | NA                         |                  |
| IKK $\epsilon$                        | Upregulation   | 342        | TBK1-II                    | 354              |
| BRCA1                                 | Mutation       | 346        | NA                         |                  |
| BRCA2                                 | Mutation       | 346        | NA                         |                  |

**Abbreviations:** SERD, selective ER degrader; SERM, SER modulator; NA, not applicable.

nervous systems.<sup>19</sup> ERs belong to the nuclear hormone receptor superfamily (or nuclear receptor family), which are highly conserved throughout evolution. These receptors are mostly present in the nucleoplasm, but may also be bound to the plasma membrane. Their early appearance in evolution contributed to the wide range of effects triggered by this pathway, such as ER regulating lipid and glucose metabolism in the liver,<sup>20</sup> whereas in cardiac tissue estrogen prevents apoptosis and necrosis of cardiac and endothelial cells.<sup>21</sup>

The main mechanism of ER activation is through estrogen (E2) binding to its receptor (ER). E2-ER complexes translocate to the nucleus and bind to EREs present in the DNA, regulating transcription of a subset of ERE-modulated genes. Typically, an ERE encompasses a 15 bp palindromic RE containing two PuGGTCA motifs, separated by 3 bp.<sup>22</sup> Binding of the E2-ER complex also results in recruitment of coactivator complexes, with histones being further acetylated, leading to chromatin remodeling and finally to recruitment of the basal transcription machinery.<sup>23</sup> More than 230 estrogen-stimulated human genes with conserved and unconserved elements have been identified, and more than 70,000 potential ERE motifs have been found through genome wide screenings.<sup>24</sup> In humans, the main estrogen hormone is 17 $\beta$ -estradiol

(E2), which binds to the ligand binding domain of the ER, a globular domain that harbors a hormone binding site and a dimerization interface and also contains coactivator–corepressor interaction capabilities. However, ERs display a ligand cavity slightly wider than their ligands, allowing them to be activated by a set of steroid hormones, environmental contaminants, xenoestrogens, phytoestrogens, and synthetic compounds with diverse structures.<sup>25,26</sup>

An alternative mechanism involving activation of ER in the absence of estrogen binding has been described,<sup>27–29</sup> in which ER activation occurs through phosphorylation by cellular kinases cross-coupling with ER signaling. Phosphorylation of ERs on both serine<sup>30</sup> and tyrosine<sup>31</sup> residues has been observed, with serine phosphorylation of the N-terminal transcriptional activation function (AF)-1 domain (which influences the receptor’s transactivation activity) and phosphorylation of ER’s serine 118 (necessary for EGF response involving the MAPK pathway).<sup>32</sup> Further signaling studies revealed a new membrane-coupled receptor, namely GPR30, which can mediate a rapid response. GPR30 (also named GPER1) is a G-protein-coupled receptor located in the cell membrane and endoplasmic reticulum that stimulates intracellular calcium mobilization, activation of phosphoinositide kinase signaling, and inhibition of cell migration.<sup>33,34</sup>

## ER variants

Two ERs are expressed in human cells: ER $\alpha$  (ER $\alpha$ 66), encoded by the *ESR1* gene, and ER $\beta$  (ER $\beta$ 1), encoded by *ESR2*. ER $\alpha$  displays six distinct domains, termed “A”–“F”, whereas ER $\beta$  has the same distribution but lacks the first “A” domain. In most cells, ER $\alpha$  and ER $\beta$  localize to the plasma membrane and to cytoplasmic organelles, such as mitochondria and the endoplasmic reticulum, exhibiting overlapping tissue distribution and functions under normal conditions.<sup>35</sup> However, ER $\alpha$  and ER $\beta$  integrate the estrogen pathway with opposing functions for some genes involved in cell proliferation.<sup>36</sup> For instance, in HeLa cells, estrogen activates ER $\alpha$ , whereas ER $\beta$  is activated by antiestrogens.<sup>37</sup> Additionally, ER $\alpha$  and ER $\beta$  have opposite actions at the cyclin D1 promoter,<sup>36</sup> where ER $\beta$  is able to inhibit cyclin D1 expression even when ER $\alpha$  is activated by saturating concentrations of 17 $\beta$ -estradiol. These opposing functions are purportedly carried out by the activation of functional domains (AF1) on both receptors. ER $\beta$  does not contain a strong AF1 domain, but contains a repressor domain that may inhibit the overall transcriptional activity of the receptor.<sup>38</sup> These studies support a potential modulatory role for ER $\beta$  in proliferation of mammary tissue induced by estrogen.<sup>36</sup>

Although ER $\alpha$  and ER $\beta$  receptors have similar structures, their splice variants are much more diverse. In ER $\alpha$ , for instance, exon skipping generates isoforms with highly variable lengths and motif composition.<sup>39</sup> The variants generated by alternative splicing could play an important role in cancer development.<sup>40</sup> Many of these variants are translated as ERs with diverse responses to hormone stimulation. For example, ER $\beta$  increases ER $\alpha$  degradation, while the isoform ER $\beta$ 2 is strongly correlated with high grade prostatic cancer and poor prognosis. Currently, a great deal of information linking splicing variants to susceptibility to disease is available, in addition to their roles in maintaining normal physiology. Different types of variants can exert diverse effects: some variants can fail to bind ligands, others display altered subnuclear localization and capacity for transcriptional activation by dimerization, while others bind to cofactors more weakly and fail to bind ERE to start transcription.<sup>41</sup>

## Genomic and nongenomic pathways

There are two main types of ER signaling pathways. The first, named genomic or classical mechanism, is initiated by activation of the ER in response to a given stimulus. ERs can be activated through binding of their specific ligand (E2) or by phosphorylation, independently of the ligand, prompting transcription of a group of genes, either by direct binding to promoter sequences or by activating complexes of transcription factors that bind to non-ERE sequences. The E2–ER complex recruits coregulatory proteins to promoter sequences, activating or inhibiting transcription, the result being determined by the type of ligand, the type of complex formed, and even by the presence of some splicing variants. Activation of this pathway from stimulus to response may take several hours.<sup>42</sup>

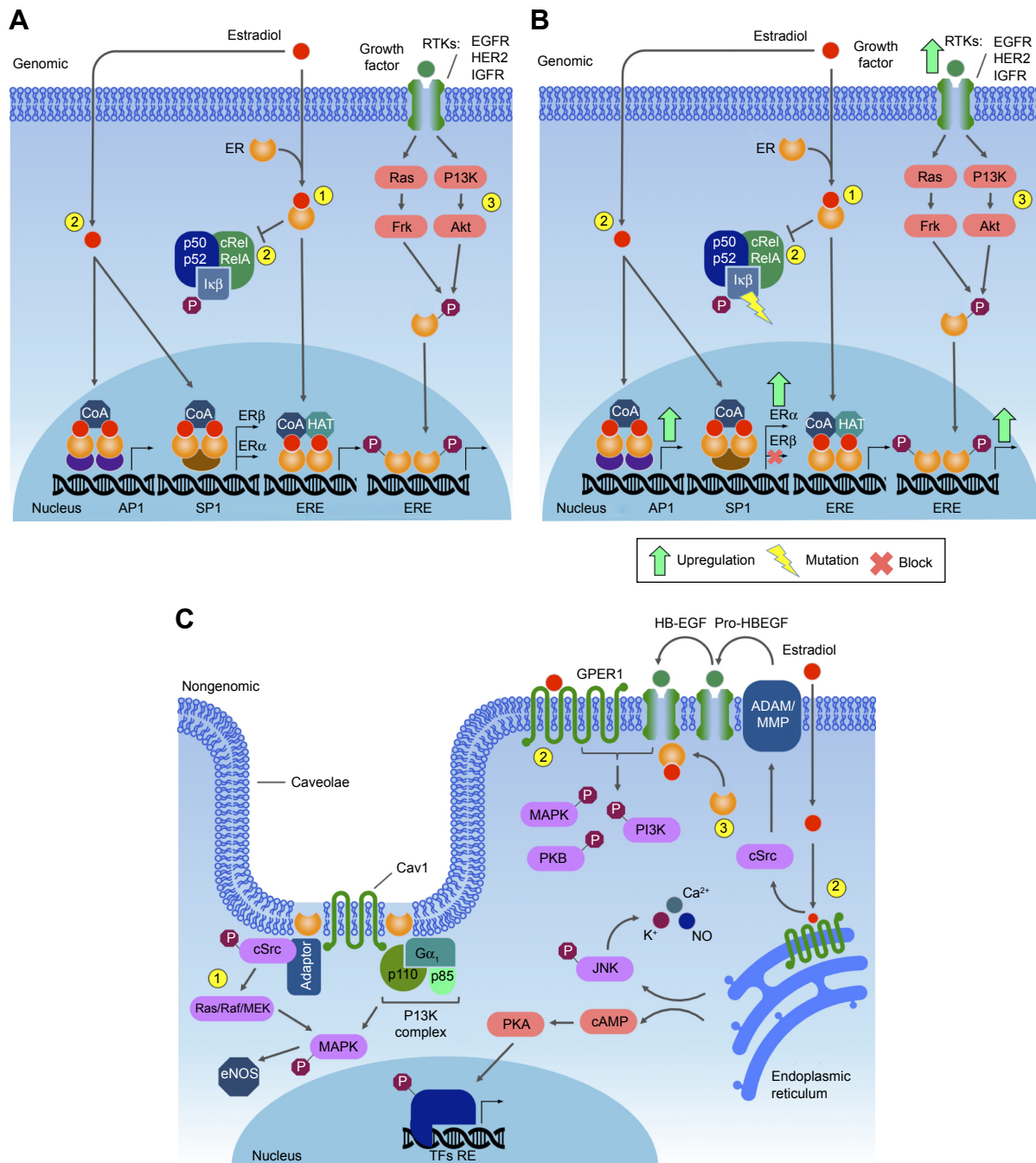
The second type of ER signaling pathway, nongenomic mechanism, triggers much faster responses, ie, within seconds to minutes. In this mechanism, ER activation on the plasma membrane or adjacent to receptors is mediated by various protein kinase cascades, generating such responses as nitric oxide (NO) release, increases in ion flux across membranes, and activation of RTK and the protein lipid kinase pathway.<sup>34</sup> Also, E2 stimulates G $\beta\gamma$  subunit protein-dependent transactivation of the EGFR–ERK signaling axis, through release of pro-HBEGF and suppression of the EGFR–ERK pathway.<sup>43</sup> The proposed mechanism involves membrane-localized GPR30, ER $\alpha$ , and ER $\beta$  associated with G-protein complexes, generating the earliest rapid signals.<sup>44–46</sup> Specific G-proteins or proteins physically associated with ER can activate growth factor receptors, such as EGFR, which triggers secondary signals, like ERK and PI3K activation, calcium, PKC, or Src kinase.<sup>47–49</sup> Most of this membrane protein



complex is associated with scaffold anchor proteins, such as caveolin 1, striatin, and SHC, generally in caveolae.<sup>50–52</sup> The outcome of these signaling pathways is the modulation of cell adhesion, migration, survival, and proliferation, thus playing an important role in the onset of cancer<sup>53</sup> (Figure 1).

## ER in mammary tissue development

The E2–ER complex has essential roles in morphogenesis in many female tissues, such as uterus,<sup>54,55</sup> ovary,<sup>56</sup> and mammary glands,<sup>57</sup> as well as male tissues like gonads<sup>58</sup> and the prostate.<sup>59,60</sup> In normal mammary gland tissue, cell division



**Figure 1** ER genomic pathway.

**Notes:** (A) E2–ER activates gene expression through direct binding to specific ERE sequences, recruiting coactivators (CoAs) and histone acetyltransferases (HATs) (1); ER and E2 modulate gene expression through interactions with other transcription factors (TFs) (2); ER ligand-independent activation through receptor tyrosine kinase (RTK) signaling (3). (B) In breast cancer, the microenvironment containing fibroblasts and inflammatory and endothelial cells has a critical role in the initiation and progression of tumors, providing growth factors, cytokines, and chemokines that activate ER and upregulate its target genes. (C) ER nongenomic pathway. ER localizes at the cell membrane in caveolae, binds Cav1 and interacts with adaptor proteins/cSrc. This complex activates MAPK and PI3K–Akt pathways (1). E2/GPER at the cell membrane and endoplasmic reticulum activates cSrc signaling, releasing HB-EGF, which binds to EGFR and activates MAPK/PI3K–Akt pathways (2). ER associates with RTKs in response to E2 binding. E2–ER recruits additional coactivator molecules, leading to the activation of RTKs and the downstream kinase pathway (3).

starts during puberty, proceeding with cyclic cell proliferation, controlled by endocrine regulation through ovarian secretion of E2 and also by GH and IGF1 to complete branching morphogenesis.<sup>61,62</sup> In adult tissue, progesterone plays an important role in mammary SC expansion.<sup>63</sup> E2, GH, and IGF1 receptors mediate pathways regulating ductal growth and morphogenesis.<sup>64</sup>

The human breast tissue epithelium is arranged hierarchically, involving the participation of SCs, which do not express ER $\alpha$  or PR, differentiated hormone-sensing ER $\alpha$ <sup>+</sup> cells, and myoepithelial cells. Morphologically, two main types of epithelial cell types may be distinguished: luminal cells lining the central lumen, where the majority of ER $^{+}$  cells are found, and the myoepithelial cells, which lay adjacently to the basement membrane.<sup>65,66</sup>

A possible mechanism for the estrogen sensitivity of the mammary tissue is that ER $\alpha$ <sup>+</sup> cells initiate proliferation by signaling in a paracrine manner. Therefore, ER $\alpha$ <sup>+</sup> cells would be required to initiate proliferation,<sup>67,68</sup> such that after estrogen stimulation, EGFs, such as Areg, are released by ER $\alpha$ <sup>+</sup> cells and trigger cell proliferation.<sup>69,70</sup> Areg is strongly induced by estrogen in mammary epithelial cells at puberty, binding to EGFR, which is located on stromal cells. This protein requires induction of the ADAM17 metalloproteinase to be released from mammary epithelial cells and reach the stromal cells, a mechanism that allows activation of stromal EGFR.<sup>71</sup>

In the suggested model, GH induces the production of IGF1 in both the liver and locally in mammary stroma and epithelium. As such, E2 and IGF1 generate the first signal to trigger proliferation, required for ductal morphogenesis. The effect of E2 on ER $\alpha$ <sup>+</sup> sensing cells then induces the release of Areg, which acts through stromal cells, recruiting additional growth factors that contribute to the rapid growth of mammary glands, and stimulating terminal end bud formation and ductal branching, which occurs at puberty.<sup>72</sup> Therefore, a paracrine circuitry model consisting of estrogen response via cross talk with the stroma and IGF1 as the primary effector downstream of E2 and GH<sup>73,74</sup> is responsible for the sensitivity of breast tissue to estrogen. In adult mammary gland tissue, progesterone has a proliferative effect, which is activated via a paracrine mechanism. However, estrogen also upregulates PRs, playing a key role in maintaining differentiated ER $\alpha$ <sup>+</sup> cells and the presence of populations of SCs in the adult mammary gland.<sup>63</sup>

## ER and breast cancer

In most cases of breast cancer, it is well known that the E2-ER complex is involved in malignant transformation

and progression. Estrogen is important in the maintenance of breast tissue; however, the mechanism by which this hormone renders quiescent epithelial ER $\alpha$ <sup>+</sup> cells into highly proliferative cells during tumorigenesis is still poorly understood. The answer may lie in the cross talk between epithelial ER $\alpha$ <sup>+</sup> and stromal EGFR signaling, since some studies show that both ER $\alpha$  and Areg are upregulated in early hyperplastic precursors of breast cancer, which together may initiate tumorigenesis.<sup>75</sup> On the other hand, estrogen withdrawal ensues during menopause. The suppressive activity of ER $\alpha$  on some growth factor receptors in the normal mammary epithelium is affected by the decrease in estrogen level during menopause, allowing for expression of growth factor receptors on ER-positive cells, thus rendering these cells potentially proliferative and cancerous. Once this occurs, ER may be activated by growth factor-stimulated TKs, and normal regulation of cell proliferation is then lost.<sup>76,77</sup>

Breast cancer may arise by different mechanisms. Overexpression of HER2 in 12%–20% of breast cancers is often due to gene amplification.<sup>78</sup> In some types of breast cancers, there are specific translocations, such as those between chromosomes 12 and 15 (t[12;15]), commonly detected in secretory carcinomas of mammary tissue,<sup>79</sup> as well as that between chromosomes 11 and 19 (t[11;19][q21;p13]), which is characteristic of breast mucoepidermoid carcinoma.<sup>80</sup> In some subtypes of ER $^{+}$  breast cancer, somatic mutations have been identified by whole genome sequencing. These mutations are present in a set of genes that impact important signaling pathways, which possibly affect ER function.<sup>81</sup> For instance, the luminal A breast cancer subtype displays mutations in *PIK3CA* (49%), *MAP3K1* (14%), *GATA3* (14%), *TP53* (12%), and *MAP2K4* (12%) and loss of *PTEN* (13%), among others. The luminal B breast cancer subtype has mutations in *TP53* (32%), *PIK3CA* (32%), *MAP3K1* (5%) and other genes.<sup>82</sup> These data reinforce the concept that breast cancer is etiologically diverse, with the current classification based on histology and morphology reflecting the heterogeneity of this disease.

An emerging group of nuclear receptors involved in key processes of mammary tissue development are orphan nuclear receptors.<sup>83</sup> These receptors share functional domains with the ER, wherein their AF1 sites are ligand-independent<sup>84</sup> and thus constitutively active. Several studies have demonstrated a correlation between the clinical outcomes of different types of breast cancer with the expression of these receptors, such as COUP-TFI,<sup>85,86</sup> NGFIB,<sup>87</sup> rROR $\alpha$ ,<sup>88,89</sup> ERR $\alpha$ , ERR $\beta$ , and ERR $\gamma$ . Due to their high degree of homology with ERs, more emphasis has been given to the study of ERRs and particularly

to  $ERR\alpha$ , a master regulator of cellular energy metabolism in both normal and cancer cells.<sup>90–92</sup> Interestingly, high  $ERR\alpha$  expression positively correlates with HER2 status and poor outcome in breast tumors, suggesting a tentative possibility for a new prognostic biomarker.<sup>93,94</sup> Recent studies found that  $ERR\alpha$  is part of the AMPK–PGC1 $\alpha$ – $ERR\alpha$  axis, a key regulator in reprogramming of cellular metabolism and cellular adaptation to metabolic stress.<sup>95,96</sup> PGC1 $\alpha$ – $ERR\alpha$  is a known repressor of folate metabolism<sup>97</sup> and one of the main pathways upregulated in cancer cells; however, several studies have associated the PGC1 $\alpha$ – $ERR\alpha$  transcriptional axis with increased tumor growth in breast cancer.<sup>97,98</sup> One possible explanation for this apparent contradiction is the number of metabolic pathways regulated by the PGC1 $\alpha$ – $ERR\alpha$  axis,<sup>99–101</sup> acting in conjunction and cross talking to achieve energy balance in response to stress and several signals in the tumor microenvironment. These results highlight the use of  $ERR\alpha$  as a putative biomarker in breast cancer, and reinforce the idea that directing specific drugs to the folate pathway in tumors overexpressing PGC1 $\alpha$ / $ERR\alpha$  is a powerful tool to improve patient prognosis.<sup>83</sup>

## Clinical targeting of the ER pathway

Overexpression and activation of ER $\alpha$  increase cell proliferation and malignant transformation of luminal-type breast cancers,<sup>75</sup> prompting the development of antiestrogen treatments. The most successful therapies for ER $\alpha$ <sup>+</sup> breast cancer have relied on synthetic molecules designed to block mainly ER $\alpha$ , such as selective ER modulators like tamoxifen, raloxifene, and toremifene, aromatase inhibitors, and selective ER degraders, such as fulvestrant. Though highly effective, these drugs have unwanted side effects in nontarget tissues, with approximately 50% of patients acquiring resistance and developing further metastases.<sup>102,103</sup> Combined drug therapies, such as tamoxifen and aromatase inhibitors, have been shown to improve disease-free survival substantially.<sup>104</sup> Expression of ER, PRs, and HER2 determines the tumor hormone receptor status. In fact, these molecular markers are valuable to determine prognosis and predict response to anti-ER $\alpha$  therapy.<sup>105</sup>

In normal breast tissue, the predominant ER is ER $\beta$ . However, ER $\beta$  levels are reduced in breast tumors, compromising the potential efficacy of targeted therapies.<sup>106</sup> Most compounds that selectively target ER $\beta$  elicit anti-inflammatory effects, but show no therapeutic effect in cancer. Alternative targets are also currently under development, such as G1 (agonist) and G15 (antagonist), specifically targeting GPR30. Although drugs targeting ER are widely

used, caution should be applied, since the mechanisms by which these drugs operate, their effects on specific tissues and cell types, and their specificity toward each receptor type, including GPR30, remain to be determined.<sup>107</sup>

## Phosphatidylinoside-3 kinase The PI3K pathway

PI3K signaling is central for a number of cellular processes related to cell growth, proliferation, motility, survival, and apoptosis. This pathway mediates metabolism by modulating both glycolytic flux and fatty acid synthesis. Activation of PI3K signaling consistently leads to cell mass accumulation and cell proliferation, as well as differentiation events by promoting protein synthesis, cell cycle progression, and actin rearrangement.<sup>108</sup> PI3K signaling also modulates autophagy, a nonapoptotic programmed cell death mechanism, as well as protein and organelle recycling into metabolic intermediates through lysosomes.<sup>108–110</sup>

PI3Ks are lipid-based protein kinases, usually divided into three distinct classes (I, II, and III). Class I is subdivided into IA and IB, according to their structural characteristics and substrate specificity. Class IA consists of heterodimers with regulatory and catalytic subunits, the former mediating activation of the latter by RTKs, G-protein-coupled receptors, and adaptor proteins, such as the Ras oncoprotein.<sup>111–113</sup> Upon activation, PI3K phosphorylates PIP<sub>2</sub> in the plasma membrane, converting it into PIP<sub>3</sub>. Dephosphorylation of PIP<sub>3</sub> is an important modulatory step in this pathway, due to the activity of the PTEN tumor suppressor, which acts as a negative regulator of this pathway.<sup>114</sup> PIP<sub>3</sub> recruits and activates Akt, also known as PKB. This activation stimulates the catalytic activity of Akt, resulting in phosphorylation of other downstream proteins that regulate cell growth, survival, and entry into the cell cycle. The Akt protein has three isoforms: Akt1, Akt2, and Akt3. During breast cancer progression, Akt1 promotes cell proliferation by S6 and cyclin D1 upregulation, while Akt2 promotes cell migration and invasion through F-actin and vimentin induction.<sup>108,113</sup> Akt3 is the less studied isoform, but is known to be mainly involved in triple-negative breast cancers, in which it may play a role in migration, invasion, and tumor growth.<sup>115</sup>

One of the major downstream effectors of the PI3K signaling pathway is a serine/threonine protein kinase complex called mTOR. mTOR is subdivided into two complexes – mTORC1 and mTORC2 – which are structurally similar but functionally distinct.<sup>116,117</sup> Autophagy is regulated by mTORC1, which can either phosphorylate or dissociate the ULK1 complex to block or initiate autophagy, respectively. When nutrients

are limited, mTORC1 dissociates from the ULK1 complex, initiating autophagy, in which PIP<sub>3</sub> plays an important role, establishing the autophagosomes and translocating the cytoplasmic material to lysosomes for degradation.<sup>109</sup> mTORC2 organizes the cellular actin cytoskeleton and regulates Akt phosphorylation<sup>116</sup> (Figure 2).

## PI3K in mammary tissue development

In vivo studies suggest that the p110 catalytic isoform p110 $\alpha$ , the catalytic subunit of class IA PI3K, is involved in duct outgrowth and branching during puberty, postpartum lactation, EGF signaling in mammary epithelial cells, and mammary gland development. The p110 $\beta$  isoform may play a regulatory role in refinement of PI3K downstream signaling, responding to insulin and EGF, while its absence induces a moderate hypermorphic mammary gland phenotype.<sup>118</sup>

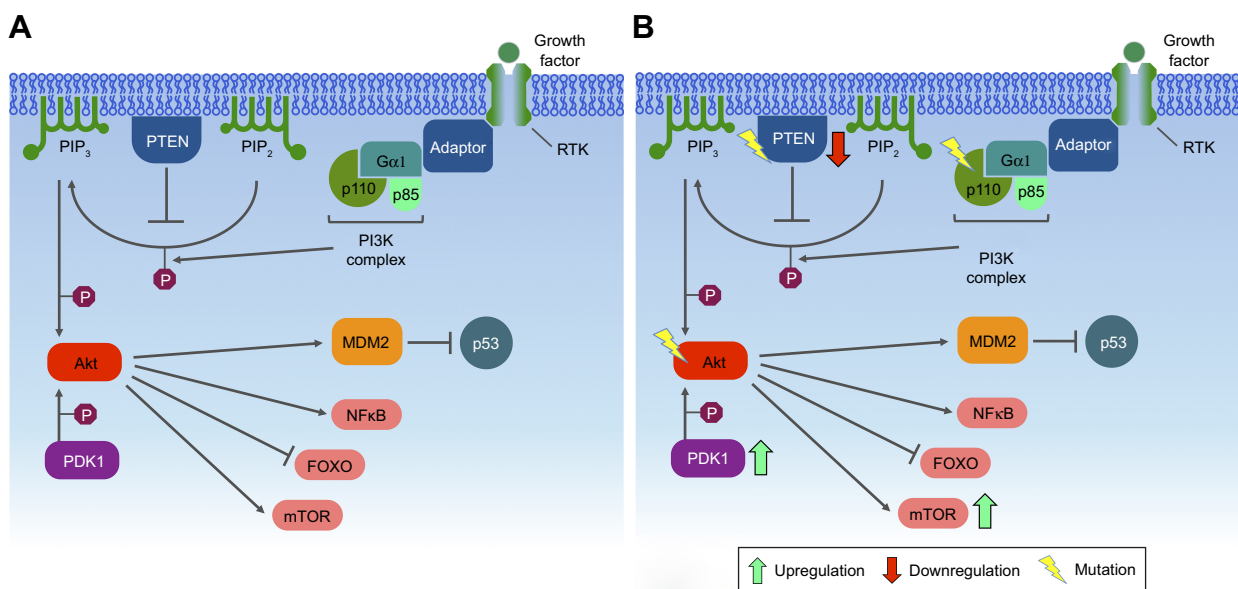
mTORC1 requires RAPTOR as a main cofactor, while mTORC2 requires RICTOR.<sup>119</sup> With mammary epithelial cells, it has been observed that Akt inhibition decreases branching and colony size, suggesting that Akt is involved in signaling growth control and branching morphogenesis dependent on RICTOR. Moreover, inhibition of mTORC2 reduces ductal branching and lengthening. Finally, it has been suggested that the mTORC2–RICTOR complex is necessary for PKC $\alpha$  signaling through the small GTPase Rac1, in order to trigger mammary morphogenesis cell survival, cell junctions, and motility.<sup>120</sup>

## PI3K and breast cancer

Mutations in *PIK3CA*, the gene encoding the p110 $\alpha$  catalytic subunit of class IA PI3K, are among the most common alterations observed in human malignancies, found in approximately 25% of breast cancers. This mutation confers a gain of function to p110 $\alpha$  and may render HER2-based therapy ineffective.<sup>117,121</sup> Most of the mutations in *PIK3CA* occur in three sites: two in the helical domain (E542 and E545) and one in the kinase domain (H1047).<sup>122</sup> Other mutations along the PI3K pathway are frequently found in human cancers, especially breast cancer. *PTEN* mutations are frequent in basal-like (67%) and HER2<sup>+</sup> (22%) breast cancers, while *AKT1* mutations are often found in luminal/HR<sup>+</sup> (2.6%) tumors.<sup>116</sup>

Notch activation may render breast cancer cells resistant to PI3K–mTORC1 inhibition, and loss of PTEN may lead to resistance to Notch inhibition.<sup>123</sup> The Notch signaling pathway is evolutionarily conserved, and mainly regulates cell fate determination during development and maintains adult tissue homeostasis.<sup>124</sup> In mammals, four Notch proteins are present (Notch1, Notch2, Notch3, and Notch4), which are defined by a large extracellular domain and a cytoplasmic domain. In particular, Notch1 is involved in breast cancer tumorigenesis and metastasis, apparently triggering EMT in epithelial cancer cells.<sup>124</sup>

With regard to the function of Akt during breast tumorigenesis, Akt1 and Akt2 regulate cell migration and invasion



**Figure 2** The PI3K signaling pathway.

**Notes:** (A) Activation of receptor tyrosine kinase (RTK) by a ligand results in phosphorylation of the receptor and adaptor proteins. The PI3K catalytic subunit phosphorylates PIP<sub>2</sub> (PtdIns[4,5]–P<sub>2</sub>), generating PIP<sub>3</sub> (PtdIns[3–5]–P<sub>3</sub>), which recruits Akt and PDKs to the plasma membrane. Akt can be phosphorylated and activated by both PIP<sub>3</sub> and PDK I. Activated Akt modulates several transcription factors. (B) In breast cancer, mutations in several PI3K pathway members impact downstream functions, including gene expression.



in different manners. While Akt2 promotes cell migration and invasion through F-actin and vimentin induction, Akt1 inhibits cell migration and invasion through downregulation of  $\beta_1$  integrin and FAK.<sup>125</sup> Akt may be directly phosphorylated and activated by  $\text{PIP}_3$  or by the putative PDK1/PDK1. Activation of  $\text{PIP}_3$  provides a docking site (containing pleckstrin homology domains) for both Akt and PDK1.<sup>113</sup> In breast cancer, PDK1 is overexpressed and amplified in approximately 20% of tumors. PDK1 phosphorylates Akt1 at Thr<sup>308</sup>, suggesting that another protein kinase, namely PDK2, is responsible for Ser<sup>473</sup> phosphorylation. However, it is still unclear whether PDK2 is an alternative kinase or a modification of PDK1 by the PDK1-interacting fragment.<sup>126</sup>

### Clinical targeting of the PI3K pathway

Several preclinical and initial clinical trials have shown great potential in targeting the PI3K–Akt pathway in cancer-related conditions.<sup>127</sup> Inhibition of the PI3K pathway has been associated with improved antitumor T-cell response and tumor angiogenesis, since: 1) the activity of p110 $\beta$  is involved in tumors displaying PTEN loss; 2) p110 $\alpha$  drives angiogenesis; 3) p110 $\gamma$ , p110 $\delta$ , and p110 $\beta$  have important functions in inflammatory cells in the tumor microenvironment; and 4) p110 $\delta$  and mTOR control important aspects of adaptive immunity, which include lymphocyte activation and differentiation.<sup>128,129</sup> For breast cancer treatment, PI3K inhibitors can be very effective, especially when combined with HER2 inhibitors, since *PIK3CA* mutations seem to be associated with resistance to these drugs. Therefore, a combinatorial treatment by inhibiting PI3K, in addition to HER2 blockade, may constitute a powerful therapy.<sup>121</sup> Targeting mutated kinases and RTKs may be interesting for drug development, considering that similar therapies have demonstrated promising results in other cancer types, such as non-small cell lung cancer.<sup>122,130</sup> LY294002 and wortmannin comprise the first generation of PI3K inhibitors and, thus lack selectivity for particular PI3K isoforms and present toxic effects. Some other drugs, such as BEZ235, BKM120, and BGT226 (from Novartis), XL765 and XL147 (from Exelixis), or SF1126 (from Semaphore), which inhibit nonselective PI3K/mTOR, have been employed in preclinical or in clinical trials. Other drugs, such as PX316 (from ProIX Pharmaceuticals), GSK690693 (from Glaxo-SmithKline), Akti1/2 and MK2206 (both from Merck), and XL418 (from Exelixis), inhibit Akt activity. Another viable target in the PI3K pathway is the mTOR node, blocked by the anticancer drugs rapamycin (also known as sirolimus [Rapamune, from Pfizer]), CCI779 (Torisel, from Pfizer),

RAD001 (Afinitor, from Novartis), and AP23573 (from Merck/Ariad).<sup>113</sup>

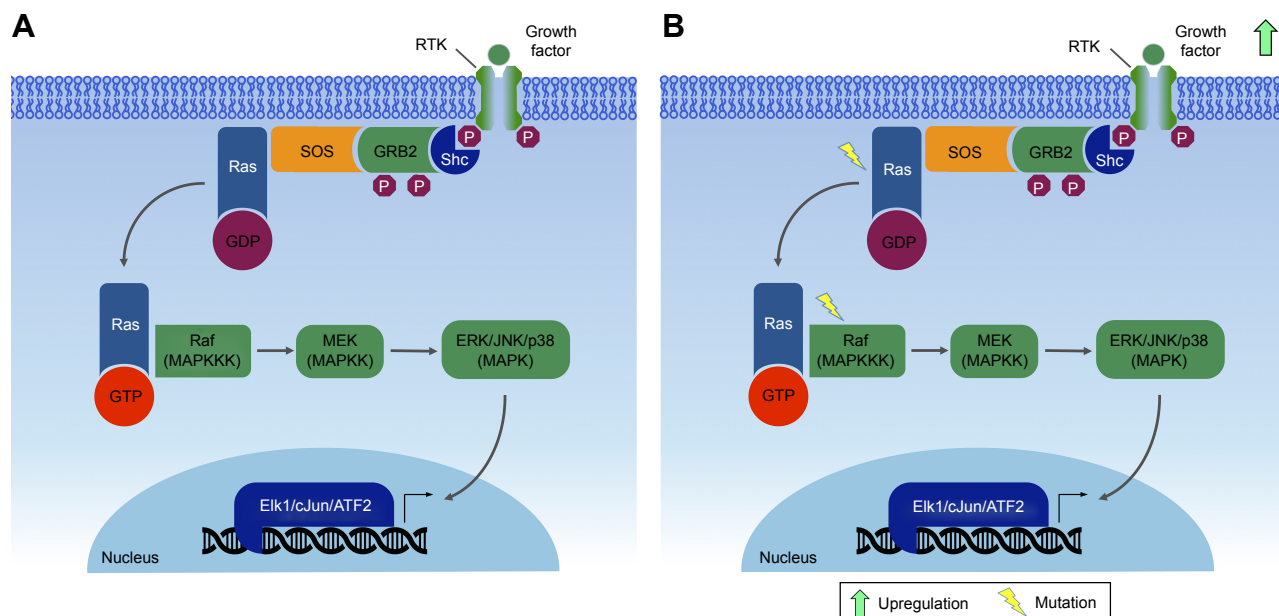
### Mitogen-activated protein kinase The MAPK pathway

MAPKs are phosphoproteins stimulated by mitogens.<sup>131</sup> Most of the signals directly involving MAPKs are evolutionarily conserved and biologically versatile, and found in animals, plants, fungi, and protists.<sup>132–135</sup> MAPK activity is regulated by a cascade composed of a MAPK, MAPKK/MKK/MEK, and MAPKKK or MEKK. The MAPK pathway may be activated by STE20 kinase or small GTP-binding proteins,<sup>136</sup> and is responsible for controlling cell survival and adaptation in response to chemical and physical stress.<sup>135,136</sup>

MAPKs are key components in the control of embryogenesis, cell differentiation, growth, proliferation, migration, and apoptosis.<sup>135,137</sup> Six groups of MAPKs have been characterized in mammals: 1) ERK1/2, 2) ERK3/4, 3) ERK5, 4) ERK7/8, 5) JNK1/2/3, and 6) the isoforms of p38  $\alpha/\beta/\gamma/\delta$ .<sup>135,138</sup> MAPKs are composed of 80 kDa homodimers containing a threonine/x/tyrosine domain, where the X represents glutamate (E), proline (P), or glycine (G), in ERK, JNK, or p38, respectively,<sup>139–141</sup> with N- and C-terminal regions of different lengths.<sup>138</sup>

Generally, the initial stimulus in this cascade comes from an extracellular signal, such as a peptide growth factor, binding to its receptor protein embedded in the plasma membrane. This binding induces the receptor, such as RTKs, to initiate an autophosphorylation process that elicits the adaptor protein SHC, which in turn binds RTK and becomes self-phosphorylated. The receptor SHC complex then recruits the GRB2 adapter protein and the SOS guanine nucleotide exchange factor, catalyzing the conversion of GDP-Ras to GTP-Ras, which leads to MAPKKK phosphorylation. MAPKKK phosphorylates MAPKK in two serine residues, Ser218 and Ser222, thereby rendering it active and capable of phosphorylating MAPK in a threonine and tyrosine residue.<sup>132–135,142–146</sup> Depending on the stimulus, phosphorylation of MAPKKK may be carried out by other small GTPases, such as Rac, CDC42, and Rho, which in turn modulate MAPK activation status downstream<sup>138,147</sup> (Figure 3).

A single MAPK cascade may lead to different cellular responses. Every MAPK is activated by specific MAPKKs (MEK1/2 for ERK1/2, MKK3/6 for p38, MKK4/7 [JNKK1/2] for the JNKs, and MEK5 for ERK5); however, each MAPKK may be activated by more than one MAPKKK.<sup>136</sup> For instance, the cascade comprising Raf1 or Mos (MAPKKK),



**Figure 3** MAPK signaling pathway.

**Notes:** (A) Specific ligands bind to the transmembrane receptor tyrosine kinase (RTK). The receptor, activated by transphosphorylation, recruits GRB2 and SOS, changing Ras-GDP to active Ras-GTP. Ras-GTP recruits and phosphorylates MAPKKK, which in turn phosphorylates MAPKK and finally MAPK. MAPK is translocated to the nucleus, which will further phosphorylate API transcription factors that will mediate expression of target genes containing a TPA DNA-response element (TRE), like cFos and cJun. (B) In breast cancer, excess extracellular growth factors or mutations in Ras and/or MAPK affect the expression of target genes.

**Abbreviation:** TPA, tetradecanoyl phorbol acetate.

MEK1/2 (MAPKKs), and ERK1/2 (MAPKs)<sup>133,142,148</sup> may induce mitogenesis, transdifferentiation (PC12 cells) and even activation of the CDC2 cyclin B complex (oocytes), depending on the cell type and the stimuli.<sup>133,149</sup>

The Raf–MEK–ERK–MAPK cascade, present in various animal cell types, can be activated by the GTPase Ras.<sup>133,142</sup> Activated Ras induces the protein kinase activity of Raf kinases. The Raf family of protein kinases is composed of A-Raf, B-Raf, and Raf1,<sup>150</sup> and each of the isoforms contains three conserved regions: CR1, CR2, and CR3. The CR3 region corresponds to the kinase domain, whereas the CR1 and CR2 regions are located in the amino-terminal end, being involved in regulation of the catalytic domain. Deletion of CR1 and CR2 generates constitutively active Raf1 mutants, which may be activated independently of Ras.<sup>150,151</sup>

## MAPK and breast cancer

Mutations in *RAS* have been observed in many tumors.<sup>152</sup> These mutations are mainly associated with the constitutive activation of ERK1/2, thus promoting tumor cell proliferation. Abnormalities in MAPK signaling affect most of the cellular processes commonly associated with the development of cancers:<sup>135</sup> independence from proliferation checkpoints, evasion of apoptotic signals, unlimited replicative potential, invading and metastatic capacities, and the ability to attract

and sustain angiogenesis. In particular, recent studies have reported higher frequency of modified cells with activated MAPK in breast cancer.<sup>153</sup>

Mutations along the MAPK pathway, which may lead to the development of cancer, are often found in Ras and B-Raf, whereas JNK and p38 appear to be poorly related to malignant transformation.<sup>135</sup> The ERK pathway has been the best studied to date, with deregulations being described in approximately a third of human cancer cases. In cancer cells, the constitutive activation of ERK signaling occurs in the early steps of the pathway, and may be due to a series of factors, such as overexpression of RTK, activating mutations in TK receptors, autocrine or sustained paracrine production of activating ligands, and mutations of *RAS* and *BRAF*. However, this amplification and/or deregulation may also occur in targeted nuclear transcription factors, such as c-Myc and AP1.<sup>135</sup>

Due to the high frequency of activating mutations, the Ras–Raf axis is suggested as a regulatory node of the pathway. Mathematical modeling predicts that activation of this node leads to general activation in the cell.<sup>135</sup> Ras GTPases control the activity of various signaling pathways, and mutations in *KRAS* and *NRAS* have already been described for several types of cancers, since they lead to inefficient GTP hydrolysis, causing Ras to be in an active state and leading

to continuous activation of the MAPK route. This deregulated Ras activation can also recruit scaffold proteins, such as KSR and SUR8/SHOC2, which modulate Raf activation by Ras.<sup>135</sup>

Human breast cancer may be subdivided into estrogen-dependent and -independent types. Estrogen typically activates growth factors which increase the levels of MAPK activity. In estrogen-independent tumors, MAPK stimulates growth upon activation by other peptide hormones.<sup>153</sup> The functions of MAPK in breast cancer appear to be complex, owing to several cellular responses that they modulate and also their interaction with different pathways (ie, ER and HER2). Generally, MAPKs are associated with good prognosis across most classifications of breast cancer, notably in those with positive ER.<sup>154</sup>

Studies demonstrate that ERK1/2 and phospho-ERK1/2 are associated with good clinicopathological status and that good prognosis may be related to their roles in inducing apoptosis. Phosphorylated JNK1/2 has also been shown to be stimulated by stress or growth factors, activating apoptosis and even augmenting cell death signaling in MCF7 breast cancer-derived cells under the influence of high estradiol levels. Finally, growing evidence suggests a role for phospho-p38 in inducing apoptosis in breast cancer. Also, MAPKs have been associated with longer survival of ER<sup>+</sup> patients.<sup>154</sup>

Activated MAPK can also phosphorylate ER, either directly or indirectly, and increase its transcriptional efficiency (an important feature of hormone-dependent breast cancer). A recent report also suggested that RSK (downstream target of MAPK) can phosphorylate ER, an effect that increases its own transcriptional efficiency.<sup>155,156</sup> Additionally, MAPK can phosphorylate PR at sites that act as ubiquitination signals, leading to degradation by the 26S proteasome.<sup>157</sup>

In the absence of estrogen and progesterone, breast cancer cells could be stimulated by growth factors (ie, EGF, EGF1, insulin, prolactin, TGF $\alpha$ , or TGF $\beta$ ), increasing MAPK activation. Cells overexpressing ErbB2 (a type of RTK) exhibit increased activated MAPK, which is mediated through ErbB2 interaction with endogenous ligands, such as EGF and neuregulin.<sup>153</sup> The tumor suppressor PTEN, with dual-specificity phosphatase activity, blocks phosphorylation of the insulin-stimulated MAPK in MCF7 cells by inhibiting IRS1 phosphorylation and inhibiting the formation of IRS1–GRB2–SOS complexes. Consequently, cell growth is suppressed and cyclin D1 downregulated, halting cell cycle progression.<sup>158</sup>

MAPKs have an important role in cell cycle arrest and sequestration of ERK in the cytoplasm. Feedback to upstream mediators is common in inhibitory signals mediated by mutated *RAS* and *RAF*. Further stimulation through this pathway is omitted by HDM2 and FOXO3. The MAPK cellular function is context-specific and cell type-specific, in order to mediate signals that can lead to diverse cellular functions. Furthermore, the function of MAPKs is affected by their cross talk and interaction with other pathways, which can influence their behavior.<sup>154</sup>

## Clinical targeting of the MAPK pathway

Since MAPK signaling regulates both physiological and pathological processes, upstream inhibition is not advised as a good method for treatment. Each MAPK could be involved in several responses; therefore, blocking an MAPK for treatment of a specific disease could be deleterious for another physiological process, while inhibiting an upstream protein could cause negative downstream effects. Sorafenib, a drug that can inhibit the Raf kinase, is approved for treatment of several cancer types.<sup>159,160</sup> However, no targeted therapy is available for other MAPKs. Many p38 blocking drugs have been tested, but so far none has reached approval for use, due to their low efficiency and/or hepatotoxicity.<sup>161</sup>

## Janus kinase/signal transducer and activator of transcription

### The JAK–STAT pathway

Several extracellular signals may cause rapid changes in the expression of specific genes. The JAK–STAT pathway is one of example of how cells are able to recognize signals to generate rapid and accurate responses. Studies of interferon-induced intracellular signaling have led to the discovery of this pathway, which relates to the major signaling mechanism used by cytokines and growth factors.<sup>162</sup> Among the main cytokines that activate this pathway are interferons, interleukins, and colony-stimulating factors. Among growth factors, such hormones as prolactin, leptin, erythropoietin, and thrombopoietin are the main activators.<sup>163</sup> Activation of this pathway stimulates important cellular events, such as proliferation, differentiation, migration, and apoptosis, as well as critical processes, such as hematopoiesis, immunodevelopment, mammary gland development, and lactation.<sup>164,165</sup>

This pathway is relatively simple, comprising a few major components: cell surface receptors, JAKs (TKs that are constitutively associated with the receptor), and STATs (latent cytoplasmic transcription factors).<sup>162</sup> JAKs are cytoplasmic TKs that participate in downstream signaling of various cell surface

receptors that have no intrinsic TK activity. In mammals, four members of the JAK family are known: JAK1, JAK2, JAK3, and Tyk2.<sup>166,167</sup> JAKs share seven domains (JH1–JH7), including: the four-point-one protein–ezrin–radixin–moesin (FERM) domain, which is important for association of JAKs with receptors; the SH2 motif, with scaffold function; the pseudokinase motif, which regulates JAK kinase activity; and the TK motif, which is catalytically active, being important for phosphorylation of receptors and STATs.<sup>168</sup>

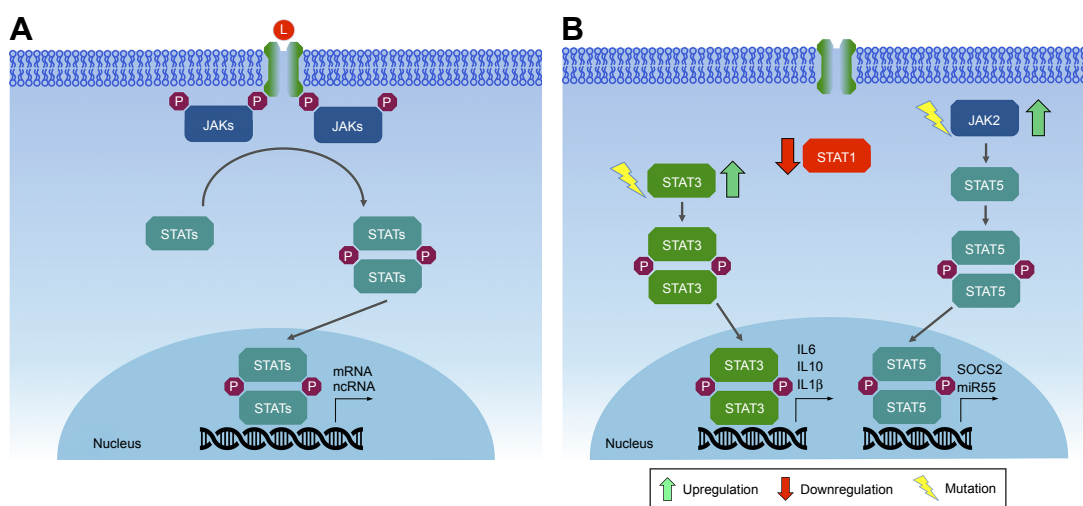
STATs are latent cytoplasmic transcription factors, which are activated upon recruitment to an activated receptor complex. To date, seven members of the STAT family have been identified in mammals: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6.<sup>163</sup> Six well-defined domains compose STAT structure, including: a conserved N-terminal domain involved in regulation of STAT activity, such as the formation of STAT tetramers and tyrosine dephosphorylation;<sup>169</sup> a coiled-coil domain, involved in receptor binding and association with regulatory proteins;<sup>170</sup> a DNA binding domain; the SH2 domain, which is important for recruitment of STATs to the activated receptor complex; and a variable C-terminal transactivation domain, which is important in transcriptional modulation of target genes. Interaction among the different types of JAK and STAT proteins will depend on the cytokine receptor that has been activated.<sup>171</sup>

Binding of cytokines or growth factors to their specific receptors on the cell surface leads to receptor dimerization and subsequent activation of JAKs by transphosphorylation (ie, JAK molecules phosphorylate each other).

Activated JAKs have the ability to phosphorylate specific tyrosine residues present in the receptor, which will then serve as anchoring sites for STATs. Anchored STATs are phosphorylated by JAKs, leading to their homodimerization or heterodimerization. Phosphorylated and dimerized STATs leave the receptor and translocate to the nucleus by an importin  $\alpha_5$ -dependent mechanism and the Ran nuclear import pathway, where they bind DNA at specific regulatory regions in promoters, activating transcription of target genes (Figure 4).<sup>172,173</sup>

## JAK/STAT in mammary tissue development

The STAT family of transcription factors participates significantly in the development of the mammary gland, since its members play important roles in the regulation of cell proliferation and apoptosis. Deletion of STAT3 results in fetal lethality, preventing further investigation into the development of the gland, although some authors believe that STAT3 is important in the self-renewal of mammary SCs.<sup>174</sup> Other members of the STAT family (1, 3, 5, and 6) have important functions in the adult mammary gland. For instance, STAT1 is phosphorylated in the virgin gland (a gland that did not undergo physiological changes) and late involution during remodeling of the gland, but not during pregnancy and lactation. STAT3 is a mediator of cell death and inflammatory signaling in gland involution, becoming phosphorylated on the day of birth and in the first 10 days of involution. STAT5 is essential for lobuloalveolar development and expression of milk protein genes, being predominantly activated during pregnancy. Finally, STAT6 induces the expression of



**Figure 4** JAK–STAT signaling pathway.

**Notes:** (A) Specific ligands bind to membrane receptors, leading to dimerization, which promotes JAK activation and transphosphorylation, which in turn phosphorylates STAT, prompting its dimerization. STAT dimers enter the nucleus and binds to specific DNA sequences, activating gene expression. (B) In breast cancer, constitutive activation of certain members of the pathway leads to the overexpression of genes that promote growth and apoptosis resistance.



cytokines and important transcription factors in the maintenance of luminal alveolar cells.<sup>175</sup>

During lactation, mammary gland cells maintain upregulated growth to produce milk through activation of STAT5. However, upon weaning, the mammary glands undergo an involution process. During involution, STAT5 is inactivated and STAT3 triggers the apoptosis process to reduce cell numbers.<sup>175</sup> One of the transcriptional targets of STAT3 is the PI3K regulatory subunits p55 $\alpha$  and p50 $\alpha$ , which in turn inhibit or downregulate PI3K/Akt survival signaling to promote apoptosis. In addition, STAT3 downregulation of Akt, which blocks activation of STAT5, might represent an additional mechanism of mammary gland involution.<sup>176–178</sup>

## JAK/STAT and breast cancer

Due to the high number of cytokines and growth factors that activate this pathway, gain-of-function, loss-of-function mutations, and polymorphisms in *JAK* and/or *STAT* genes are associated with several human diseases. Constitutive activation of this pathway may arise by several mechanisms, including production of autocrine/paracrine cytokines, activating mutations (point mutations that generate amino-acid substitution) of receptors, JAKs and/or other upstream oncogenes, which then activate STATs.<sup>179</sup> Due to the important roles in regulating cell proliferation and survival, as well as in inflammation, it is not surprising that most JAKs and STATs are also implicated in tumorigenesis of breast tissues, acting either as oncogenes or as tumor suppressors.

Somatic mutations in members of the JAK family are rare in cancer.<sup>180</sup> Despite this, somatic mutations in *JAK1*, *JAK2*, and *JAK3* have already been described in breast cancer samples.<sup>181,182</sup> Recent research shows the possible involvement of JAK2 and TYK2 in the development of breast cancer. Studies on epithelial mammary cells show that constitutive activation of JAK2 caused by the V617F point mutation generates hyperactivation of STAT5, leading to increased proliferation and resistance to cell death. Transgenic mice expressing *JAK2*<sup>V617F</sup> presented an accelerated tumorigenic process.<sup>182</sup> *Tyk2*<sup>-/-</sup> mice inoculated with breast cancer cells show increased tumor growth and metastasis compared to wild-type mice, suggesting a possible tumor suppressor role, mediated in part by myeloid-derived suppressor cells.<sup>183</sup>

A great deal of evidence indicates the involvement of STAT1, STAT3, STAT5, and STAT6 in the formation, progression, prognosis and prediction of breast cancer.<sup>177</sup> Depending on the physiological context, STAT1 may act as an oncogene or as a tumor suppressor. Approximately 45% of ER<sup>+</sup> and 22% of ER<sup>-</sup> breast cancer cases exhibit low levels

of STAT1 in neoplastic cells, while high levels of STAT1 are detected in benign breast tissue adjacent to the tumor. This may indicate that *STAT1* is repressed during breast tumor progression.<sup>184</sup> Conversely, high *STAT1* expression is associated with metastasis and drug resistance.<sup>185</sup> It appears that in postmenopausal ER<sup>+</sup> breast tumors, STAT1 acts as a tumor suppressor, while in ER<sup>-</sup> tumors or ER<sup>+</sup> premenopausal malignancies, STAT1 promotes tumor progression.<sup>177</sup>

STAT3 is constitutively activated in a wide range of solid tumors, including breast cancer.<sup>186</sup> Its aberrant activation may promote invasion and metastasis, in addition to regulating the inflammatory response in breast tumorigenesis. Human studies have shown that activation of STAT3 is frequently observed in primary breast cancers, being associated with poor prognosis and tissue invasion.<sup>187,188</sup> Constitutive activation of STAT3 appears to affect the tumor microenvironment through secretion of various cytokines, such as IL-10, IL-6, IL-1 $\beta$ , by tumor cells, which stimulate nontumor cells, T-helper (T<sub>H</sub>)-17 cells, and tumor associated macrophages to secrete even more cytokines, thereby creating a positive feedback loop. This promotes growth and differentiation of tumor cells. STAT3-directed secretion of IL-10 by tumor cells also results in inhibition of antitumor immunity, eg, by inhibiting maturation of dendritic cells.<sup>189</sup>

Like STAT3, STAT5 is also constitutively activated in breast cancer, but is considered weakly oncogenic in breast cancer mouse models. According to the data currently available, activated STAT5 may promote tumorigenesis, but it is not a genuine proto-oncogene, at least in breast cancer. Activated STAT5 promotes tumorigenesis by expanding the population of mammary alveolar cells, which have been suggested to be especially susceptible to tumorigenesis.<sup>190</sup> Finally, STAT6, which is important in the balance between T<sub>H</sub>1 and T<sub>H</sub>2 cells, also affects tumor progression, facilitating evasion of the immune system by tumor cells.<sup>189</sup> T lymphocytes can differentiate into either T<sub>H</sub>1 cells or T<sub>H</sub>2 cells, depending on the cytokines secreted in their environment. T<sub>H</sub>1 cells identify tumor antigens and trigger an immunoresponse, whereas T<sub>H</sub>2 cells display oncogenic potential, promoting invasion and tumor metastasis. STAT6 is essential for the differentiation of T lymphocytes in T<sub>H</sub>2 cells mediated by IL4.<sup>177</sup>

## Clinical targeting of the JAK–STAT pathway

Mounting data showing the relevance of the JAK–STAT pathway in diseases related to the immune system and various types of cancers render the members of this pathway

attractive as therapeutic targets. The effectiveness of targeting JAK–STAT signaling has been demonstrated in clinical trials in patients with solid tumors.

## JAK inhibitors

The first studies with JAK inhibitors (ie, tyrphostin AG490) led to inhibition of recurrent leukemia B-cell growth in vitro and in vivo.<sup>191</sup> In the early 2000s, another JAK inhibitor (pyridone 6) was introduced by Merck, with activity against all members of the JAK family in vitro; however, this compound did not present significant results in vivo.<sup>192</sup> Analysis of its crystallographic structure showed that pyridone 6 binds to adenosine triphosphate pocket in the JH1-kinase domain of the JAK2 active conformation. This information served as the basis for the development of various JAK inhibitors described thus far.<sup>193</sup>

Several selective JAK inhibitors are currently in clinical trials for the treatment of solid tumors. These inhibitors focus mostly on two members of the JAK family: JAK1 and JAK2. Ruxolitinb (Novartis), which targets both JAK1 and JAK2, is one of the most promising compounds, used in various clinical trials (Phase I, II, and III) for a wide variety of solid tumors (breast, lung, gastric, colon, colorectal, and pancreatic cancer). Another interesting inhibitor targeting JAK1 and JAK2 (mometotinib; Gilead Sciences) has also been tested for lung, colon, and pancreatic cancers. The INCB047986 and INCB39110 inhibitors (Incyte Corp.), which specifically target JAK1 by blocking its phosphorylation, are also in advanced phases of clinical trials.<sup>194</sup>

## STAT inhibitors

Constitutive activation of STATs is well described in several types of cancer.<sup>195–197</sup> STATs are important transcription factors for pathology progression, by controlling genes with key roles in cell proliferation and modulation of the tumor microenvironment.<sup>198</sup> Therefore, the STAT family is an increasingly attractive therapeutic target in cancer. However, great challenges persist in developing STAT inhibitors, since their blockade is much more complex than blocking kinases, such as JAKs.<sup>179</sup> One of the challenges lies in target specificity, which may generate off-target effects, resulting in undesired side effects.<sup>199</sup> Another challenge is redundancy in the action of different STATs. For example, selective STAT3 blockade, which is critical for IL-6 downstream signaling, may not be fully effective since IL-6 can also act through *STAT1* signaling.<sup>200</sup>

Despite these limitations, a wide range of molecules that mainly inhibit STAT3 and STAT5 have been developed and

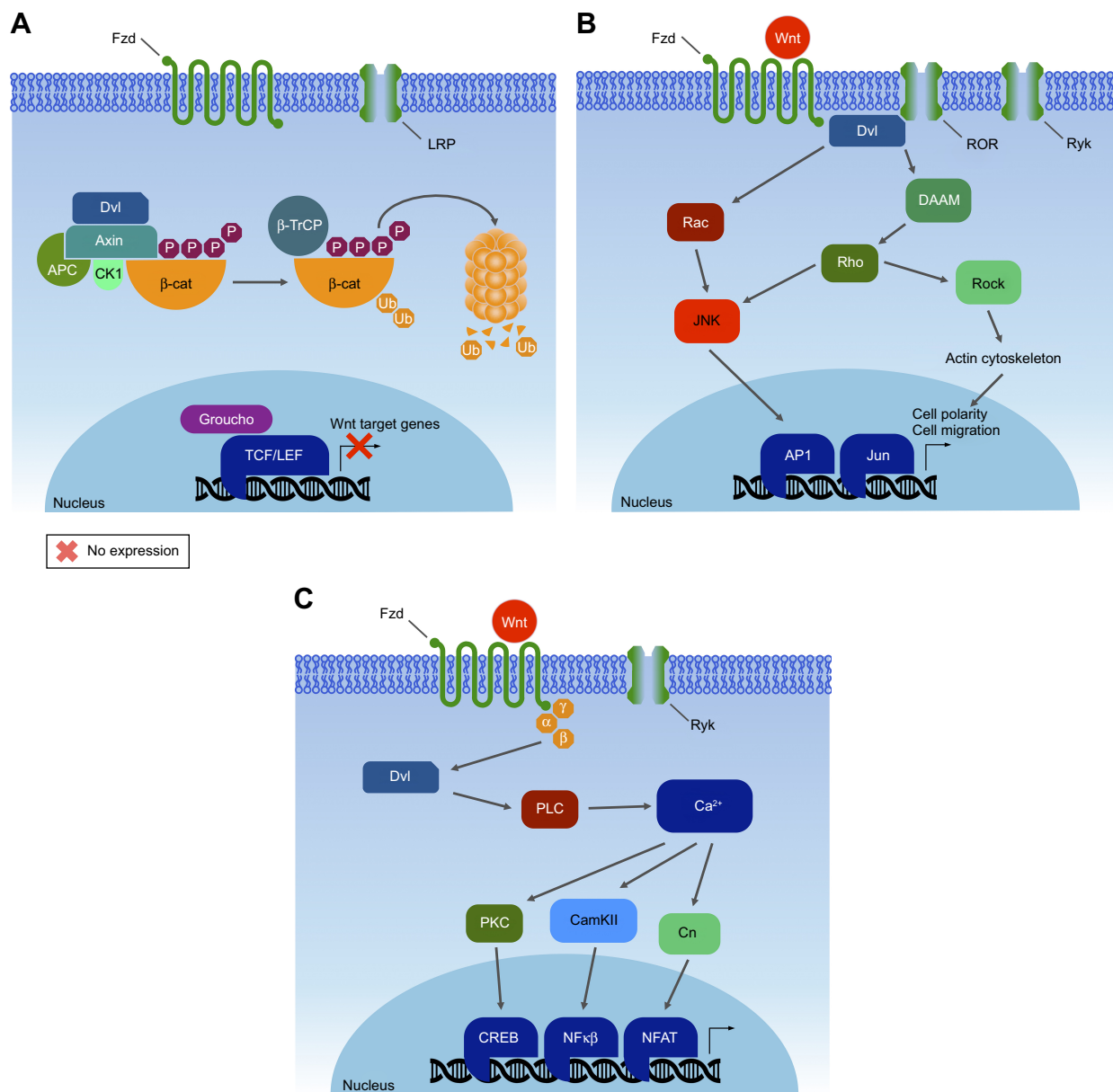
tested both in vivo and in vitro. These include peptidomimetics, small-molecule inhibitors, and oligonucleotides.<sup>201</sup> As an example of peptidomimetics, CJ1383, a STAT3 inhibitor, has shown promising results in two breast cancer cell lines with high levels of phosphorylated STAT3.<sup>202</sup> Several small inhibitory molecules targeting the SH2 domain in STAT3 and STAT5 should also be mentioned. LLL12, S31201 (both from Biovision, Inc.) and SF-1066, which inhibit STAT3 activity in different ways, have shown important results using the MDA-MB-231 breast cancer line as a model.<sup>203,204</sup> FLL32 and IS3295, which inhibit STAT5, have also shown promising in vitro results in several breast cancer cell lines, such as MDA-MB-231, SUM159, and SK-BR-3.<sup>205</sup> Finally, as an example of nucleotide-based inhibitors, siRNAs and G-quartets (guanine-rich oligonucleotides that form inter- or intramolecular four-stranded structures) against STAT3 have shown positive results, based on studies using the MCF7 and MDA-MB-231 breast cancer cell lines.<sup>206–208</sup>

## Wingless-type MMTV-integration-site family

### The Wnt pathway

Human and most mammalian genomes harbor 19 *WNT* genes, falling into 12 evolutionarily conserved *WNT* subfamilies.<sup>209</sup> Wnt proteins are secreted ~40 kDa cysteine-rich glycoprotein ligands, activating a complex mechanism of signal transduction via multiple pathways: the canonical  $\beta$ -catenin-dependent pathway and several noncanonical  $\beta$ -catenin-independent pathways.<sup>210</sup> These Wnt cascades play an important role in embryonic development processes, as well as in carcinogenesis.<sup>211</sup> The noncanonical Wnt signaling pathway, which operates independently of  $\beta$ -catenin-mediated transcription (Figure 5A), is separated into the Wnt planar cell polarity and Wnt/Ca<sup>2+</sup> signaling branches.<sup>212</sup> Moreover, this pathway appears to function independently of transcription (Figure 5B and C).

The canonical pathway was first identified and delineated from genetic screens in *Drosophila melanogaster*, and intensive studies in worms, frogs, fish, and mice have led to the identification of a basic molecular signaling framework.<sup>213</sup> Briefly, the canonical Wnt pathway is defined by the translocation of  $\beta$ -catenin into the nucleus, where it acts as a coactivator of transcription. Wnt ligands bind to an Fzd and a member of coreceptors LRP5/6. Intracellular signaling proceeds via the Axin and Dvl proteins (Figure 6A). These in turn lead to inactivation of the  $\beta$ -catenin destruction complex, which causes  $\beta$ -catenin to be stabilized and relocated into the nucleus. Within the nucleus,  $\beta$ -catenin forms a complex with



**Figure 5** Wnt pathways.

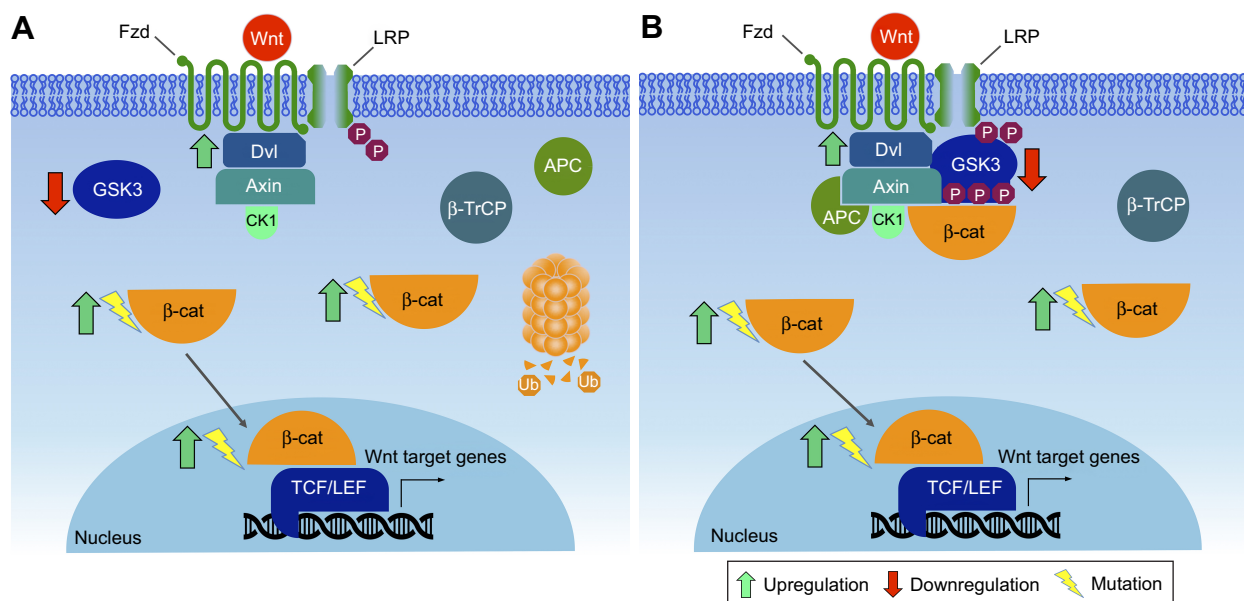
**Notes:** (A) Planar cell polarity (PCP) Wnt pathway in "off" state, with no ligand bound to Fzd receptor leads to β-catenin proteasome degradation. (B) In the noncanonical β-catenin-independent PCP Wnt pathway, Wnt binds to the Fzd receptor and membrane coreceptors ROR and Ryk. These receptors activate the cytoplasmic signaling protein Dvl, which interacts with Rac and DAAM. Rac activates JNK, while DAAM activates Rho, which activates Rock to regulate cellular cytoskeletal arrangements and actin polymerization. (C) Wnt signaling can also go through the alternative Ca<sup>2+</sup>-dependent β-catenin-independent pathway, where signaling is mediated by Dvl, G proteins, PKC, CamKII and Cn (calcineurin).

the TCF/LEF transcription factors, triggering the expression of specific target genes.<sup>214,215</sup> This transcriptional activity determines cell fate decisions, survival, and proliferation. The precise targets may vary among cell types, but in some cases can include the oncogenes *c-Myc* and *cyclin D1*.<sup>216</sup> In the new model, Axin stabilizes the destruction complex in both the presence and absence of WNT, and β-catenin is degraded through phosphorylation-mediated recognition by β-TrCP in the intact complex.<sup>212</sup> This allows newly synthesized β-catenin to accumulate in the cytosol before nuclear

translocation (Figure 6B). Despite the fact that major pathway components have been characterized, the function of Wnt signaling within the context of cancer biology is intriguingly complex and remains only partially understood.

## Wnt in mammary tissue development

Several components of Wnt signaling have been documented during various stages of morphogenesis of the mammary gland, including a wide range of Wnt ligands, receptors, downstream effectors, and DNA-binding proteins.<sup>212</sup> Functionally, it is



**Figure 6** Canonical Wnt signaling pathway.

**Notes:** (A) Wnt binds to the Fzd receptor and induces the binding of Axin to LRP. The proteasome decouples, allowing β-catenin to stabilize. Accumulated β-catenin translocates to the nucleus, where it binds TCF sites to promote the expression of target genes. (B) A new model of Wnt signaling in which Wnt binds to Fzd receptor and recruits a large protein complex composed of Dvl, Axin, and β-catenin, among others. Subsequently, β-catenin enters the nucleus to regulate gene expression. In breast cancer, both Dvl and β-catenin can be overexpressed due to different mutations, leading to upregulation of a series of target genes.

clear that canonical Wnt signaling is necessary for the initiation of mammary development. Dkk1 expression prevents localized expression of all mammary placode markers.<sup>217</sup> Wnt6 is initially expressed on the surface ectoderm as a broader band surrounding the Wnt10-expressing mammary lines.<sup>209,218</sup> In addition, Wnt4 mediates progesterone-induced ductal side branching in early pregnancy.<sup>219</sup> Contrarily, the expression of Axin, which acts to destabilize β-catenin, causes defective alveolar formation during pregnancy.<sup>220</sup> β-Catenin also plays important roles in mammary development, such as cell adhesion, signal transduction, and regulation of gene expression, which are essential for mammary SC biology during mammary development.<sup>221</sup>

Noncanonical Wnt signaling has also been shown to take part in the negative regulation of mammary epithelial outgrowths, adding another level of complexity.<sup>209</sup> For example, loss of Wnt5a during other polarized morphogenic events increases cytoplasmic and nuclear β-catenin and accelerates ductal outgrowth.<sup>222</sup> Importantly, Wnt5a is an essential mediator of TGFβ, suggesting that low thresholds of β-catenin signaling are maintained during pubertal ductal morphogenesis through TGFβ and Wnt5a antagonism.

## Wnt and breast cancer

Given the importance of Wnt signaling for adult SC biology, it is not surprising that Wnt pathway mutations are observed

frequently in cancer, most notably in tissues that normally depend on Wnt for self-renewal or repair.<sup>223</sup> Mutations including β-catenin, Axin, or other Wnt pathway components, which result in β-catenin accumulation, are found in breast tumorigenesis.<sup>224–226</sup>

Wnt signaling is activated in over 50% of breast cancer and linked to reduced overall survival.<sup>227</sup> Deregulated Wnt signaling leads to increased SC renewal, proliferation, migration, and survival.<sup>228</sup> Studies have shown that molecular subtypes of breast cancer exhibit different levels of Wnt pathway activity.<sup>229</sup> Solid human tumors, including those of the breasts, prostate, liver, and ovaries, have increased levels of cytoplasmic and nuclear β-catenin, indicating overexpression of Wnt signaling.<sup>230,231</sup> In mice, overexpression of Wnt1, Wnt3, or Wnt10 leads to mammary carcinomas.<sup>209,232</sup> Increased expression of Dvl1, a cytosolic positive regulator of Wnt signaling, is prominent in primary breast cancers.<sup>233,234</sup>

Investigations have shown that noncanonical Wnt members, such as Wnt5a receptors, are connected to breast cancer metastases,<sup>235</sup> as well as to shorter overall survival of breast cancer patients.<sup>183,236,237</sup> Immunohistochemistry expression profiling in 90 triple-negative breast cancer specimens has shown that low levels of Wnt5a expression are associated with positive lymph node metastasis, enhanced cell motility, Ki67 proliferation, and significantly worse recurrence-free survival.<sup>238,239</sup> Modulation of receptor



activation could be another factor for hyperactive Wnt signaling in breast cancer.<sup>209</sup> An alternative splicing of LRP5, which removes the coding region of LRP5 that interacts with the secreted Wnt signaling repressor Dkk1, has been reported in parathyroid and breast cancer.<sup>240</sup>

Metastasis is a trademark of late-stage cancer and a main challenge to therapy. A main adaptive change of tumors during therapy is EMT.<sup>241</sup> EMT is a known example of epithelial plasticity that is important in cancer metastasis.<sup>242</sup> During tumor progression, EMT allows benign tumor cells to infiltrate the surrounding tissue and metastasize to distant sites.<sup>243</sup> Activation of canonical Wnt signaling stabilizes Snai2 by inhibiting GSK3 $\beta$  activity and initiates EMT transcriptional programs in breast cancer cells.<sup>244</sup> Another candidate gene that regulates EMT is *ASPP2*, which encodes a protein that binds and inhibits the N-terminal phosphorylation of  $\beta$ -catenin, leading to its stabilization. Decreased expression of *ASPP2* leads to EMT and is correlated with low survival in hepatocellular and breast cancer.<sup>242</sup>

Identification of novel biomarkers would be a promising approach for developing new diagnostic and therapeutic strategies. Markers that can predict the site of metastasis are scarce, and there are few reports on the utility of evaluating Wnt signaling members in the diagnosis of cancer.<sup>245</sup> Research has suggested Dkk1 as a diagnostic biomarker for a wide variety of cancers, including breast cancer, implicating the therapeutic potential of anti-Dkk1 antibody to neutralize the activity of Dkk1 function for cancer cell invasion and growth.<sup>246</sup> The majority of cancer patients presented elevated Dkk1 levels compared to healthy controls, and thus confirmed previous data supporting the usefulness of Dkk1 as a serological biomarker of cancer.

Wnt1-expressing mouse mammary epithelial cells show transcriptional upregulation of COX2; however, it is not clear whether this is due to direct regulation of the COX2 promoter by  $\beta$ -catenin.<sup>247</sup>  $\beta$ -catenin is expressed in the nucleus, cytoplasm, and/or mesenchyme of 36.1% of breast cancer patients.<sup>248</sup> Therefore,  $\beta$ -catenin status might serve as a predictive biomarker in breast cancer.

## Clinical targeting of the Wnt pathway

Despite significant effort, there are no drugs currently approved for clinical use in breast cancer, which mainly target members of the Wnt pathway. However, a few small-molecule groups that target modulators of Wnt-related genes has emerged. Among these, the leading group are the so-called Porcupine inhibitors (LGK974 [Novartis]).<sup>249</sup> Porcupine is a membrane-bound *O*-acetyltransferase that is

required for palmitoylation of Wnt ligands, a necessary step in the processing and secretion of Wnt ligands.<sup>249</sup> LGK974 is now in clinical Phase I trials for melanoma, breast cancer (triple-negative), pancreatic adenocarcinoma, colorectal cancer, and head and neck cancers.

## Transforming growth factor beta The TGF $\beta$ pathway

TGF $\beta$  was first isolated in 1982 in a search for secreted autocrine growth factors capable of transforming normal fibroblasts into malignant cells, which can proliferate in the absence of normal growth controls.<sup>250,251</sup> The purified factor promoted wound healing by stimulating production of extracellular matrix factors and vascularization. At the same time, growth inhibitory effects of purified TGF $\beta$  were clearly demonstrated,<sup>252</sup> leading to the paradox of TGF $\beta$  playing a role in both promoting and inhibiting cell growth.<sup>253</sup> TGF $\beta$  is expressed in nearly all tissue, and the cellular response to TGF $\beta$  is highly dependent on the different contexts. The role of TGF $\beta$  has been most often studied in models for immunosuppression and inflammation, extracellular remodeling, cell proliferation, differentiation, survival, and invasion, all of which are paramount in cancer onset and progression.<sup>254</sup>

TGF $\beta$  signal transduction is carried by canonical and noncanonical pathways. In the canonical pathway, the TGF $\beta$  extracellular ligand binds the TGF $\beta_2$ -membrane receptor complex, which contains a cytoplasmic serine/threonine kinase domain that in turn phosphorylates receptor-regulated SMADs (homologue of *Drosophila* MAD), notably SMAD2 and SMAD3, to transduce the activating signal. SMAD2/3 dimerizes with the common mediator SMAD4, and this activated complex translocates into the nucleus to participate as a coactivator or corepressor of targeted gene transcription,<sup>255</sup> leading to the propagation of a diverse variety of TGF $\beta$ -induced responses in their specific contexts. TGF $\beta$  also triggers the Snail family of transcriptional repressors, which includes Snai1, Snai2/Slug, Twist1, ZEB1, and ZEB2.<sup>256</sup>

## TGF $\beta$ in mammary tissue development

Because the ubiquitously expressed TGF $\beta$  provokes many different phenotypes in different tissues, attributing specific outcomes to TGF $\beta$  is a complex exercise that largely depends on cell context and timing. Much of the insight into the role of TGF $\beta$  in mammary gland development has been achieved through experiments using ectopic TGF $\beta$  treatment of explants and transgenic mouse models. Early experiments showed that implanted slow-release pellets of

TGF $\beta$  inhibited mammary ductal branching and proliferation at the mammary end buds.<sup>257,258</sup> Consistent with this observation, transgenic mice expressing TGF $\beta$  driven by an MMTV enhancer/promoter showed suppressed mammary ductal tree development, though lactation was unaffected and no spontaneous tumors emerged in this model.<sup>259</sup> The converse inhibition of TGF $\beta$  using dominant-negative transgenic mice resulted in increased ductal tree side branching and proliferation.<sup>260</sup> In heterozygous TGF $\beta$  mice, which express <10% of normal TGF $\beta$  expression levels, mammary ductal and alveolar development is accelerated,<sup>261</sup> consistent with a fundamentally inhibitory role for TGF $\beta$  during mammary development. When TGF $\beta$  expression was directed specifically to alveolar cells by WAP gene regulatory elements, lobuloalveolar development was impaired and milk production inhibited.<sup>262</sup> Transgenic transplantation experiments revealed that the mammary epithelium itself was defective, ruling out possible *trans*-signaling effects.<sup>263</sup> Mammary explants treated with TGF $\beta$  also show suppression of milk production.<sup>264</sup> After lactation, the involution process of the mammary gland correlates with TGF $\beta$  expression and other apoptotic regulatory genes.<sup>265,266</sup> An inhibitory role for TGF $\beta$  in ductal morphogenesis and alveolar development and function and a promotional role for TGF $\beta$  in mammary gland involution and remodeling have been clearly established; however, mechanisms and downstream intracellular factors remain unclear.

## TGF $\beta$ and breast cancer

In cultured cells, TGF $\beta$  is a potent inducer of EMT, a prerequisite for metastasis and cancer progression. EMT precedes invasion of the vasculature, transport in circulation, penetration of the basement membrane of a distal tissue, and finally metastatic tumor formation. During the complex, stepwise process of EMT, changes in tissue architecture correspond to changes in cellular functions. Epithelial cells lose cell–cell adherens junctions and apical–basal polarity. The resulting mesenchymal cells acquire a spindle-like morphology, with increased migration and invasive potential. Expression of specific structural and differentiation genes changes as well, such as from cytokeratins and E-cadherin in epithelial cells to vimentin and N-cadherin in mesenchymal cells.

Exposure of mammary carcinoma cells to exogenous TGF $\beta$  in the absence of inhibitory antibodies increases cell invasion potential in migration assays and leads to lung metastasis in rodents.<sup>267</sup> Immunohistopathological analysis of human breast carcinomas reveals higher levels of extracellular TGF $\beta$  protein, especially at the advancing edges of

infiltrating mammary duct carcinomas and in lymph node metastases.<sup>268,269</sup>

TGF $\beta$  has been shown directly to promote EMT in breast cancer in both cell culture assays and mouse models. Exogenous TGF $\beta$  induces Ras-transformed mammary epithelial cells into EMT and secretion of TGF $\beta$  in these cells in an autocrine loop, and maintains the mesenchymal state.<sup>270</sup> TGF $\beta_1$  and TGF $\beta_3$  ligands have been identified in a screen for genes that cooperate with ErbB2/HER2 in activating EMT in a mammary epithelial cell migration assay. Addition of ectopic TGF $\beta$  recapitulated this migration in a dose-dependent manner.<sup>271</sup> Transgenic mouse models expressing both activated ErbB2/HER2 and TGF $\beta$  have also revealed modulation of the invasiveness and metastasis of mammary tumors in vivo that was dependent on the expression or repression of TGF $\beta$ .<sup>272–274</sup>

TGF $\beta$  affects EMT via downstream canonical SMAD-dependent pathways, as well as through noncanonical signaling and cross talk with other pathways.<sup>255</sup> In mammary epithelial cells, the *TWIST1* and *SNAIL* genes are induced by activated SMADs, and subsequently repress transcription of key genes involved in maintaining epithelial identity and integrity, notably E-cadherin, provoking an EMT transition.<sup>275,276</sup> Snail protein expression is found in duct-infiltrating breast carcinomas, presenting lymph node metastases and is inversely correlated with the grade of tumor differentiation.<sup>277</sup> Knocking down *TWIST* inhibits metastases of mammary carcinomas to the lung, and high levels of *Twist* expression are found in highly invasive infiltrating lobular carcinomas: breast cancers that have lost E-cadherin expression.<sup>278</sup>

A clear role for noncoding RNA in regulating TGF $\beta$ -induced EMT in breast cancer has been established.<sup>279,280</sup> Several members of the miR200 family (miR-200f) play a tumor suppressor role by inhibiting the Snail family transcriptional repressors *ZEB1* and *ZEB2*.<sup>281–283</sup> High expression of miR200f sequences targets the TGF $\beta$  pathway genes *ZEB1/ZEB2*, *SMAD2*, *SMAD5*, *SNAIL*, and others, increases E-cadherin expression, reduces cell motility, and restores an epithelial phenotype to mammary carcinoma cells in vitro.<sup>282,284</sup> Conversely, *ZEB1* and *ZEB2* are capable of suppressing miR200f clusters, creating a negative feedback loop with TGF $\beta$  promoting EMT and miR-200 antagonizing.<sup>281</sup> A similar feedback loop has been established between miR34 and Snail in a breast carcinoma cell line.<sup>285</sup>

Breast carcinomas exhibit a spectrum of both epithelial and mesenchymal phenotypes, and this nonbinary mix of features in tumors has given rise to the notion of partial EMT, describing an intermediate state where conversion to either

epithelial or mesenchymal states is possible and reversible.<sup>286</sup> Such plasticity between EMT states complicates our understanding of *in vivo* cellular processes during metastatic invasion, as the context of each specific tumor microenvironment often determines the outcome. The advent of molecular gene expression profiling has led to a narrowing identification of the cells of origin of breast cancer and suggested new ways to classify them. This research has intersected with studies on EMT, mammary SCs, and TGF $\beta$ .

A subpopulation of breast cancer cells with increased tumorigenic potential has been isolated by cell sorting using cell surface markers. These CD44<sup>+</sup>/CD24<sup>-</sup> cells were called tumor-initiating cells, due to their higher potency in initiating tumors when xenografted into immunocompromised mice.<sup>287</sup> A gene expression profile revealed that the TGF $\beta$  pathway genes were specifically expressed and activated in this CD44<sup>+</sup>/CD24<sup>-</sup> cell population. Furthermore, the gene expression signature of CD44<sup>+</sup>/CD24<sup>-</sup> cells resembled that of normal mammary SCs and putative breast cancer SCs (CSCs) more than they did other subpopulations of sorted breast cancer cells.<sup>288</sup>

Gene expression signatures from the CD44<sup>+</sup>/CD24<sup>-</sup> tumor-initiating cell population and a mammary SC population were compared in cells forced to undergo EMT, and found to share specific expression of SC markers, as well as EMT mesenchymal markers. SC-like properties, such as ability to undergo multipotent tissue differentiation, were observed in both populations.<sup>289</sup> An autocrine–paracrine feedback loop maintains the mesenchymal, stemlike state in these tumor-initiating cells.<sup>290</sup> From this point forward, the concept of a breast CSC or breast tumor-initiating cell (BTIC) was proposed, describing a distinct subpopulation of cells with SC-like self-renewing properties, expressing mesenchymal markers, including those involved in TGF $\beta$  signaling, which are capable of effecting tumor progression through invasion, migration, intravasation, and metastasis.

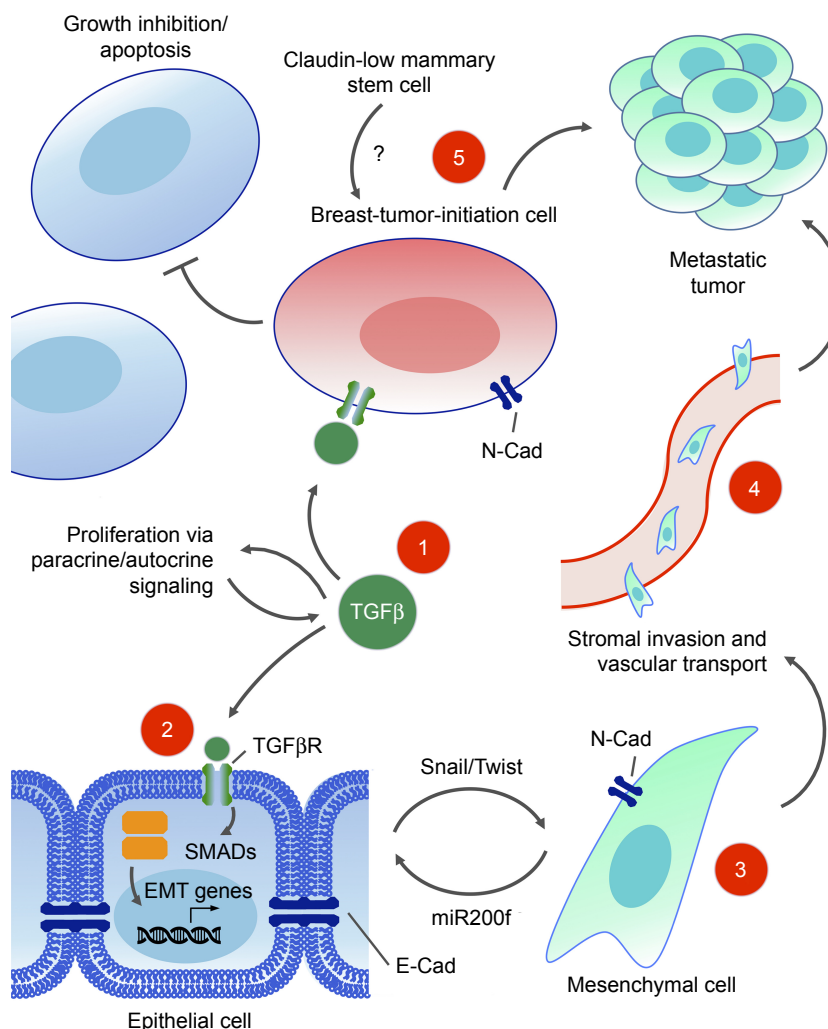
Sorting of cell populations by flow cytometry and grouping by functional gene signature profiles has allowed researchers to begin reconciling apparent contradictions in the growth-promoting vs -inhibiting effects of TGF $\beta$  signaling. Instead of a temporal switch from a cytostatic role of TGF $\beta$  toward a tumorigenic role in EMT and metastasis, both activities can take place in different cells of the same tumor cell population. CD44<sup>+</sup>/CD24<sup>-</sup>/claudin-low subpopulations of tumor and normal mammary epithelial cells respond selectively to exogenous TGF $\beta$  treatment by expansion of their BTIC or basal/SC population, whereas the BTICs of other sorted populations remain static or are reduced.<sup>291</sup> In ER<sup>+</sup> breast cancers, differing TGF $\beta$ /SMAD3-driven gene expression

signatures are generated that are able to uncouple the tumor suppressive effects of TGF $\beta$  in patient cohorts with good clinical outcomes from the tumor-promoting activities of TGF $\beta$  with poorer outcomes.<sup>292</sup> In the near future, greater knowledge about specific gene signatures from individual tumors will be informative for the choice of therapy, such as employing TGF $\beta$  antagonists or not. The epigenetic landscape will play an increasingly important role in determining TGF $\beta$ -induced phenotypes within specific cell contexts and cancer subtypes. The epigenomes of BTIC-promoting vs BTIC-suppressing breast cancer cells differentially determine the subset of target genes that can be bound and activated by TGF $\beta$ /SMAD3, adding another layer of complexity to context-dependent signaling by TGF $\beta$  (Figure 7).<sup>293</sup>

## Clinical targeting of the TGF $\beta$ pathway

TGF $\beta$  has become a popular target for drug development in cancer therapy. In addition to EMT and growth inhibition, TGF $\beta$  signaling is also involved in normal tissue homeostasis, extracellular matrix regulation, and immunoresponse modulation. With such highly pleiotropic activities of TGF $\beta$ , greatly influenced by specific contexts of cells, tissues, and architecture in complex processes, developing specific therapies to target TGF $\beta$  for a single disease presents a formidable challenge.<sup>294</sup> The attraction of targeting TGF $\beta$  would be to antagonize its EMT-promoting activities and BTIC-promoting properties, as well (perhaps) as to reduce interstitial fluid pressure to improve the efficiency of drug delivery.<sup>295</sup> Reducing TGF $\beta$  ligands or TGF $\beta$  signaling is seen as the goal, rather than direct ablation of TGF $\beta$ -inducing or -responding cells, so the efficacy of a therapy can only be assessed in a systemic *in vivo* setting, rather than via cytotoxicity screens on cells *in vitro*.

Two therapies against metastatic breast cancer have entered clinical trials. Fresolimumab, a humanized inhibitory antibody directed against the TGF $\beta$  ligand, is currently in a Phase II trial (NCT01401062) and administered in conjunction with radiotherapy. Eli Lilly has developed the drug LY2157299 (galunisertib), which targets the TGF $\beta_1$  receptor.<sup>296</sup> This small-molecule inhibitor is also administered in conjunction with radiotherapy, and is currently recruiting participants (NCT02538471). LY2157299 has been able to inhibit TGF $\beta$  signaling and consequently tumor formation in a human-derived mouse model of glioblastoma.<sup>297</sup> In another promising study, the TGF $\beta$  inhibitor LY2157299 was used in parallel with inhibitory antibodies against TGF $\beta_2$  receptors and SMAD4 RNAi. Following chemotherapy with the taxane paclitaxel, these TGF $\beta$  inhibitors blocked tumor reinitiation in a mouse



**Figure 7** TGF $\beta$  signaling in breast cancer progression

**Notes:** TGF $\beta$  modulates cell proliferation through autocrine and paracrine signaling (1). TGF $\beta$  binds its receptor on the target cell, signaling through intracellular SMAD to activate transcription of target genes, notably those that effect epithelial–mesenchymal transition (EMT) (2). Snail/Twist family of transcription factors promote transition to the mesenchymal state, corresponding to a shift from epithelial markers, such as E-cadherin and claudin, to mesenchymal markers, such as N-cadherin and vimentin. miR200f noncoding RNAs antagonize expression of Snail/Twist to promote the epithelial state (3). Migrating mesenchymal cells acquire invasive properties, such as intravasation, vascular survival, and extravasation, and effect a mesenchymal–epithelial transition to contribute to metastatic tumors (4). In breast cancer, TGF $\beta$  signaling is active in a subpopulation of CD44<sup>+</sup>/CD24<sup>−</sup>/claudin-low breast tumor-initiation cells, which share properties with normal mammary stem cells. These cells express mesenchymal markers, and are enriched after chemotherapy, promoting recurrence of metastatic tumors (5).

model of triple-negative breast cancer by suppressing the expansion of BTICs.<sup>298</sup> Such therapies combining TGF $\beta$  inhibitors with chemotherapeutic drugs show the most promise, as TGF $\beta$  activation in BTICs may promote drug resistance and cytotoxic chemotherapeutic drugs may enrich drug-resistant TGF $\beta$ -dependent BTIC populations, which contribute to tumor reinitiation.<sup>299–301</sup>

## Nuclear factor- $\kappa$ light-chain enhancer of activated $\beta$ -cells The NF $\kappa$ B pathway

NF $\kappa$ B was originally discovered as a nuclear factor that specifically binds to a 10-base-pair DNA sequence (5′-GGGA CTTTCC-3′) within the enhancer of the Ig $\kappa$  light chain

of activated  $\beta$ -cells.<sup>302</sup> Currently, NF $\kappa$ B is largely known to be involved in cell cycle regulation, immunoresponse, inflammation, proliferation, and cell death. In mammals, the NF $\kappa$ B transcription factor family is composed of five members, divided in two classes based on the sequences of the C-terminal domain. Members of one class – the NF $\kappa$ B proteins NF $\kappa$ B1/p105 and NF $\kappa$ B2/p100 – have long C-terminal domains that contain multiple copies of ankyrin repeats, which inhibit these molecules until they are processed by either limited proteolysis or arrested translation into p50 and p52, respectively.<sup>303</sup> Although p52 and p50 lack the transcription activation domains, they can positively regulate transcription through heterodimerization with members of the second class: the Rel subfamily, composed



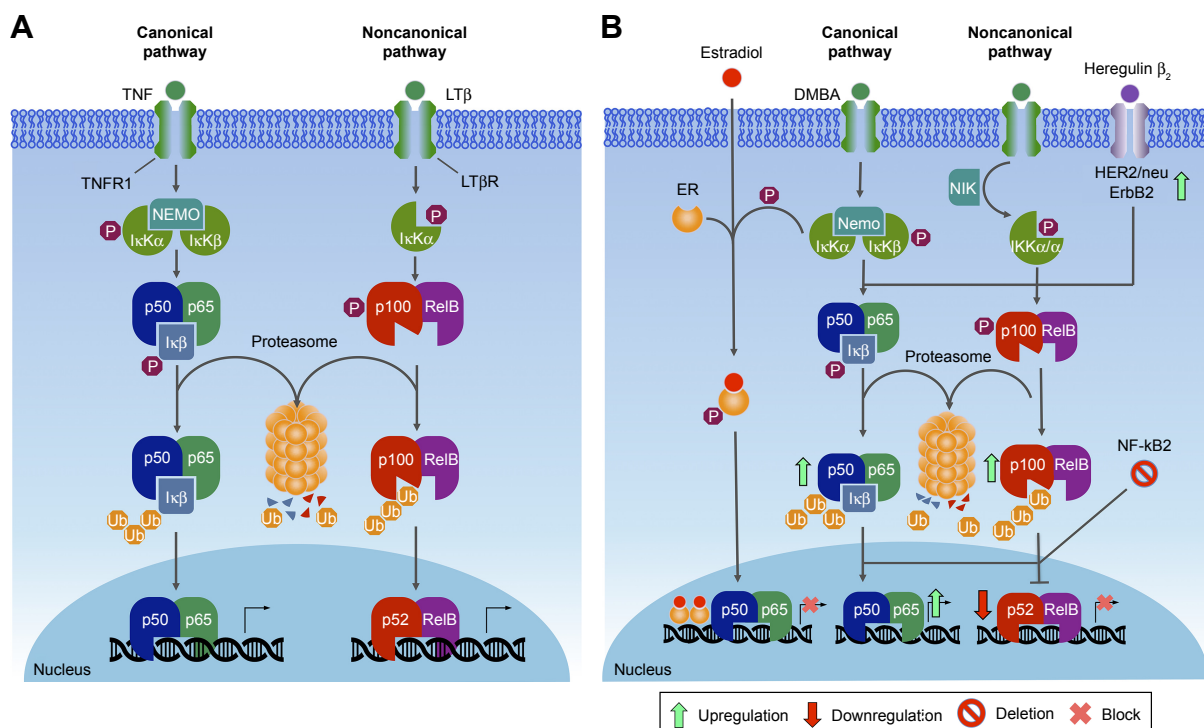
of cRel, RelB, and RelA (p65), which contain transcription activation domains that confer the ability to initiate transcription.<sup>304</sup> They all share an Rel homology region, which mediates specific DNA binding to the NFκB consensus sequence and dimerization and interaction with IκB inhibitory molecules.<sup>305–308</sup>

Prior to stimuli, NFκB dimers are bound to inhibitory molecules of the IκB protein family (eg, IκBα, IκBβ, IκBγ, IκBε), which keep NFκB complexes inactive in the cytoplasm and block their binding capacity toward DNA.<sup>309</sup> The classical (or canonical) NFκB pathway is induced by inflammatory cytokine signaling, DNA damage, or infectious agents. This stimulation leads to activation of the IκB kinase (IKK) complex (composed of IKKα/IKK1 and IKKβ/IKK2 and the scaffolding protein IKKγ/NEMO),<sup>310,311</sup> which phosphorylates IκBα and then allows the E3 ubiquitin ligase βTrCP to promote polyubiquitination and further proteasome-dependent degradation of IκB.<sup>312–314</sup> As a result, liberated NFκB rapidly enters the nucleus, where it can activate the expression of specific genes that contain DNA binding sites for NFκB (κB enhancer sites).<sup>315</sup> An alternative (or noncanonical) pathway of NFκB activation is activated

by signals transmitted by a subset of immunorelated receptors, including BAFFR, LTβR, CD40, RANKL, TNFR2, and Tweakr.<sup>316</sup> This alternative induction mechanism leads to activation of NFκB inducing kinase (NIK), which predominantly phosphorylates and activates IKKα. Activated IKK phosphorylates p100, resulting in its ubiquitination and partial processing to p52.<sup>317</sup> Following processing, alternate NFκB dimers (mainly composed of cRel and p52) are released to translocate further to the nucleus and act as transcription factors, thereby promoting expression of NFκB-related target genes. Nevertheless, binding of NFκB to the respective κB sites does not ensure transcriptional initiation, which in fact requires the interaction of NFκB with a few mediators, such as transcriptional adaptors and coactivator proteins like CBP and its paralogue p300, for assembly of the transcription machinery<sup>318</sup> (Figure 8).

## NFκB in mammary tissue development

Expression of the NFκB subunits, such as p50, p52, p65 (RelA), and RelB, as well as the prototypical inhibitor IκBα, follows a consistent pattern during mouse mammary tissue development, being typically high during



**Figure 8** NFκB pathway.

**Notes:** (A) The canonical pathway starts with binding of TNF to its receptor, activating the IKK complex, which phosphorylates IκBα, promoting its polyubiquitination and further proteasome-dependent degradation. Free NFκB rapidly enters the nucleus, where it can activate the expression of specific genes, and noncanonical pathway induction leads to the activation of NIK, which phosphorylates and activates IKKα, phosphorylating p100, resulting in its ubiquitination and partial processing to p52. (B) In breast cancer ER<sup>+</sup> tumors, IκBα phosphorylation is inhibited by estrogen in the canonical pathway. In contrast, some carcinogens like 7,12-dimethylbenz[a]anthracene (DMBA) increase NFκB. In the noncanonical pathway, the deletion of the NFκB2 gene leads to inhibition of target gene expression, while at the same time it promotes an increase in canonical pathway activity.

pregnancy, diminished during lactation, and elevated again in breast involution.<sup>319–323</sup> NFκB acts as a negative regulator of β-casein gene expression during pregnancy in mice by interfering with tyrosine phosphorylation of STAT5, blocking a premature differentiation of the gland.<sup>320</sup> Ablation of IκBα in the mammary epithelium results in hyperplasia and increased lateral ductal branching in virgin mice, suggesting that NFκB modulates proliferation, branching, and normal structural development of the mammary epithelium during early postnatal morphogenesis.<sup>324</sup> IKKα<sup>AA/AA</sup> mice bearing a serine–alanine mutation that prevents its activation, fail to develop lobuloalveolar tissue properly, which is caused by impaired RANKL-mediated induction of cyclin D1, which is encoded by an NFκB target gene.<sup>325</sup> Furthermore, a deletion of the gene for IKKβ in the mammary gland reduces apoptosis and delays involution, suggesting a proapoptotic role for IKKβ,<sup>326</sup> whereas its constitutive expression leads to a reduction β-casein levels, accompanied by detection of cleaved caspase 3 during lactation and involution.<sup>327</sup> In immortalized cultures of KIM2 cells, lactogenic hormone deprivation leads to increased NFκB activity, suggesting a survival role for NFκB in mammary involution.<sup>321</sup> The p65–p300 complex is associated with modulation of inflammatory responses 48 hours after weaning.<sup>328</sup> High levels of nitric oxide (NO) are detected during the first hours of involution, when NFκB binds to the NOS2 promoter to modulate its transcription.<sup>329</sup> Collectively, these data demonstrate the importance of NFκB signaling in the development of normal mammary glands.

## NFκB and breast cancer

Abnormally high NFκB activity is a clinical hallmark of chronic inflammation, being detected in a vast number of cancers. However, this information has to be taken with caution, since NFκB may act either as a tumor suppressor or a tumor promoter, depending on the type of cancer.<sup>330,331</sup> The role for NFκB as a tumor promoter arises from its aberrant activation and nuclear localization as a result of defects in regulation of the pathway, loss of negative feedback mechanisms, resistance to cell death, and influence of the tumor microenvironment.<sup>331</sup> However, other observations suggest that NFκB can also inhibit tumor growth. Tumor suppressor proteins, such as p53 and ARF, can induce the association of NFκB subunits with HDAC1 corepressor complexes.<sup>332</sup> This results in NFκB-dependent repression of target gene expression, providing a model through which NFκB subunits can facilitate apoptosis and cell cycle arrest and thus function as tumor suppressors themselves. This suggests a

dual function for NFκB during tumor progression, inhibiting tumor growth, but as further mutations lead to a loss of tumor suppressor expression, the oncogenic functions of NFκB become unleashed, contributing to tumorigenesis.<sup>330</sup> The tumor inflammatory microenvironment maintains NFκB constitutively active in most tumor cells, and NFκB has been found to control multiple cellular processes in cancer, such as inflammation, transformation, proliferation, angiogenesis, invasion, metastasis, chemoresistance, and radioresistance. Therefore, NFκB suppression may have a positive impact by inhibiting the growth of tumor cells.

Though NFκB is required for normal mammary gland morphogenesis, its deregulated activation has been linked to tumor progression via stimulation of cell proliferation and survival and angiogenesis pathways and metastasis, ultimately driving breast carcinogenesis. In vivo and in vitro experiments in female rats, mice, and human mammary epithelial cell lines treated with a carcinogen, such as 7,12-dimethylbenz[*a*]anthracene, showed elevated activity of NFκB prior to malignant transformation and tumor formation.<sup>333,334</sup> Elevated NFκB expression has been widely reported in human breast cancer cell lines and tissues, with abnormal constitutive expression of both canonical and noncanonical NFκB subunits, such as cRel, p65, p50, and p52.<sup>335–338</sup> Several reports have observed a positive correlation between NFκB activation and the ErbB2 receptor, also known as HER2/Neu.<sup>338–340</sup> In addition, NFκB is predominantly activated in ER<sup>−</sup> and ER<sup>−</sup>/HER2<sup>+</sup> breast cancers, suggesting its importance in specific classes of breast cancers.<sup>335,340</sup> Progression of ER-dependent to ER-independent breast cancer phenotype is correlated with an increase in NFκB activity. In an E2-independent breast ductal carcinoma cell line (MCF7/LCC1) model, constitutive activation of NFκB was observed prior to the loss of ER expression.<sup>341</sup> In ErbB2-expressing cell lines, administration of the ErbB2 activator heregulin β<sub>1</sub> led to increased NFκB activation, whereas hereceptin, an anti-ErbB2 monoclonal antibody, blocked this activation and led to apoptosis.<sup>340</sup> Phosphorylation of a tumor suppressor protein that regulates NFκB activation (CYLD) by the breast cancer oncogene kinase IKBKE<sup>342</sup> promotes cell transformation.<sup>343</sup> In vitro assays have revealed that constitutive NFκB activity in breast cancer cells stimulates osteoclastogenesis by upregulating GM-CSF. Blocking NFκB suppressed osteolytic lesion development through osteoclastic bone resorption in vivo.<sup>344</sup>

Together with oncogenic *RAS*, NFκB activity is required for protection of mammary epithelial cells from TGFβ-induced apoptosis. This protection is a prerequisite for these

cells to undergo EMT toward an invasive, metastatic tumor phenotype.<sup>345</sup> In a triple transgenic model in which NFκB activity can be inhibited by doxycycline treatment in defined windows during *PYMT*-induced tumorigenesis, inhibition of NFκB signaling leads to increased tumor latency and decreased tumor burden.<sup>327</sup>

*BRCA1*-deficient breast tumors exhibit aggressive behavior and are associated with poor survival.<sup>346</sup> Within *BRCA1*-deficient mammary glands, there is a subset of cells named luminal progenitor cells, which can form 3D colonies in a hormone-independent fashion.<sup>347</sup> In these cells, constitutive NFκB (cRel/p52) activation due to DNA damage caused by progesterone leads to hormone-uncoupled proliferation, increasing the risk of genomic instability and transformation.<sup>348</sup>

Self-renewing breast CSCs are key players in perpetuating tumor maintenance and in treatment resistance and relapse. Interestingly, in HER2-dependent tumors, the proportion of CD44<sup>+</sup> cells (marker for SC population) dramatically decreased upon NFκB suppression, indicating that this transcription factor is important in the maintenance of progenitor cell expansion.<sup>349</sup> Another study showed that repression of CD44, a cell surface glycoprotein, via NFκB inhibition consequently decreased proliferation and invasiveness of breast cancer cells.<sup>350</sup> Silencing of NFκB2/p100 in mammary cancer cell lines led to loss of p100 and p52 and to context-dependent effects on tumorigenicity of these cells.<sup>351</sup> In 4T1 mammary carcinoma cells, increased activity of NFκB (which induces EMT and CSCs) was a result of p100 loss, whereas in N202.1A mammary carcinoma cells, CSCs and tumorigenicity were positively regulated by p52, suggesting an opposite role for this protein in regulation of breast CSCs, and indicating that inhibiting p100 processing may be a potential therapeutic strategy to suppress CSC activity in a subset of breast tumors.<sup>351</sup>

## Clinical targeting of the NFκB pathway

Targeting the NFκB pathway seems to be a reasonable objective in view of the numerous implications of this pathway in diverse diseases, including breast cancer. Combinatorial therapeutic strategies, targeting members of the PI3K/Akt and MAPK pathways that lead to NFκB activation, have already been employed in several clinical trials.<sup>352</sup> Association between ErbB2 expression and NFκB activation and the inverse correlation found between the latter and ER status may be exploited in human breast cancer therapy.<sup>353</sup> Other agents, such as specific IKK inhibitors, are under investigation. Inhibition of TBK1, a noncanonical IKK,

with TBK1-II, a drug that efficiently inhibits TBK1 and IKKε, suppresses growth of human HER2<sup>+</sup> breast cancer cells and induced cellular senescence.<sup>354</sup> Inhibition of IKKs sensitizes cells to the cytotoxic effect of doxorubicin in different breast cancer cell lines.<sup>355</sup> In addition, inhibition of NFκB DNA binding is a good putative approach to inhibit NFκB activity, since this would prevent transactivation of prosurvival and antiapoptotic downstream targets, being highly selective. More than 780 compounds described to have NFκB inhibitory activity<sup>356</sup> could be used in combinatorial therapy for breast cancer. Compounds such as the proteasome inhibitor bortezomib have undergone clinical trials in breast cancer patients with some success.<sup>357,358</sup> However, unselective pathway disruption potentially leads to adverse effects, such as immunosuppression, which could be particularly harmful in cancer patients, who are likely to be immunocompromised due to previous treatments.<sup>359</sup>

## Signaling integration (crosstalk) within breast cancer

Breast cancer is heterogeneous in nature, due to a slew of aberrations at the genomic and molecular levels affecting various signaling pathways. To understand the integrative role of these pathways in different physiological and pathological conditions, it is necessary to take into consideration their complex cross talk. The best example of cross talk in the ER signaling pathway is the signalosome, a large protein complex present at the membrane. Upon activation by HER2, ERK1/2 phosphorylates ER, thus increasing its sensitivity for its ligands or leading to ligand-independent activation. In addition, ER coregulators can be modified by kinases, thereby indirectly affecting ER activity.<sup>107,360,361</sup> Also GPR30, which colocalizes with ERα in the membrane, collaborates in transmission of signals to downstream kinase cascades.<sup>45</sup>

The importance of signaling cross talk in cancer is also illustrated by the influence of estrogen signaling in EMT. Cross talk between ERα and several EMT regulators, such as Snail and Slug, leads to increased EMT.<sup>362</sup> Additionally, ER signaling can mediate delocalization of E-cadherin and cytoskeleton reorganization via the ER–Src–PELP1–PI3K–ILK1 pathway. Therefore, this cross talk can affect breast cancer progression, leading to metastasis via EMT and contributing to breast cancer cell motility.<sup>102</sup> Most of the nongenomic mechanisms in the ER pathway are implicated in breast cancer metastasis, with ERK and Akt phosphorylation being involved in breast cancer cell migration and Src and ILK1 kinases playing critical roles in cell invasion and metastasis.<sup>361</sup>

PI3K signaling also exerts downstream control on other transcriptional factors, which include members of the FOX protein family (FOXO1, FOXO2, FOXO4, and FOXO6) and NFκB. In addition, PI3K signaling can inhibit p53 activity by a mechanism that involves the E3 ubiquitin protein ligase MDM2, regulating the cell cycle, inflammation, apoptosis, and DNA repair.<sup>122</sup> LKB1, a kinase located upstream of AMPK, negatively regulates mTORC1 signaling by tuberous sclerosis 1 or 2.<sup>116,117</sup> PKC (activated in the PI3K pathway) has the potential to phosphorylate Raf1. PAK1 stimulated by PI3K and/or CDC42 pathways can also phosphorylate Raf1. Upon stimulation by PI3K, Akt can phosphorylate and inhibit both B-Raf and Raf1.<sup>153,363</sup>

NFκB-dependent transcription is not only tightly controlled by positive and negative regulatory mechanisms but also closely coordinated with other signaling pathways. Since activation depends on IκB degradation, the IKK complex is the gatekeeper of NFκB signaling, representing a critical node for interaction within parallel signaling pathways. Other upstream molecules, such as receptor interacting proteins and TNFR, are critical to IKK activation, but also signal other pathways. Thus, TNFR-associated factors represent a central point of divergence for activation of NFκB and AP1 transcription factor pathways.<sup>364</sup>

MAPKs, JNK, ERK1/ERK2, and p38 promote activation of AP1, and activated AP1 affects cell survival, apoptosis, and stress responses. Another molecule, RIP1, has been described as being involved in activation of the PI3K–Akt pathway through not only NFκB-dependent negative regulation of expression of the mTOR kinase but also through NFκB-independent downregulation of the PI3K antagonist PTEN.<sup>365</sup> The IKKβ-induced degradation of p105 may potentially influence several additional pathways, since IKKβ can also phosphorylate Dok1, an Ras GAP-associated TK substrate and cell growth inhibitor, leading to inhibition of ERK1 and ERK2, demonstrating the context dependence of IKKβ-mediated MAPK regulation.<sup>366</sup> JAKs can phosphorylate MAPK, PIK3, and TGFβ associated receptors, thereby creating a site for anchoring proteins displaying SH2 domains in their structure. Upon phosphorylation, these proteins are able to stimulate a signaling cascade in their respective pathways.<sup>367</sup>

It has been shown that ER activation can inhibit TGFβ-induced transcriptional activity and cell migration. Several lines of investigation have suggested that ERα is a major modifier of the TGFβ signaling pathway, with ER and TGFβ pathways being able to promote breast cancer metastasis.<sup>368</sup> Moreover, it is important to consider that the cross talk between Notch, Wnt, and SHH signaling pathways, as well

as their role in regulating TICs, may also modulate breast cancer onset and progression.<sup>369</sup>

## Conclusion

Clearly, the risk for breast cancer is sexually dimorphic. In fact, breast cancer is about 100 times less common among men than in women. Still, the molecular role of sex hormones modulating breast cancer risk remains elusive. Elucidation of the function of ER in breast cancer cells should provide critical new insights into the function of these receptors. Expanding the vision of the entire network involving ER may lead to better design of specific therapies to treat breast cancer, with fewer side effects. It should also provide a better understanding of the mechanisms behind antiestrogen resistance. In vitro and in vivo models, clinical trials, and innovative treatments should contribute to better understanding of the function of each type of hormone receptor.

Besides the classic hormone signaling mechanisms, the pathways discussed in this review are shown to modulate central cellular functions that are critical in the onset and progression of breast cancer. The PI3K signaling pathway is important for mammary morphogenesis and mammary gland involution; however, alterations in the PI3K pathway are very common in several diseases, including Parkinson's, obesity, type 2 diabetes, and different cancer types. Since several dysregulations in key nodes of this pathway are known to be associated with various disease states, the ability to pinpoint a specific alteration and to understand its functional relevance would allow for more accurate choice of treatment, with minimal side effects. Direct and indirect involvement of the MAPK pathway has been demonstrated for the development of breast cancer, participating in promotion of cell proliferation or rendering other pathways more active. JAK–STAT is one of the main signaling pathways that promotes communication of the extracellular medium with the cell nucleus. Wnt signaling regulates cell differentiation, proliferation, and SC pluripotency, and thus deregulation of Wnt signaling may lead to cancer development. TGFβ plays a dual role in breast cancer progression by either its tumor suppressing or tumor enhancing effects. NFκB represents a central factor in inflammation, stress response, and cell death, being recognized as an important element in several steps of cancer initiation and progression, activated by a large variety of stimuli, and displaying several downstream targets, thereby generating broad and complex feedback loops.

Several aspects of regulation of these pathways remain to be clarified, especially regarding their cross talk with other



pathways, feedback, tumor microenvironment interactions, cell metabolism, risk factors, and response to drug therapy. However, it is clear that all of these pathways act as a large orchestrated network, and that a broad scope needs to be taken in planning future therapy strategies and designing prognostic tools. In this context, an increasingly detailed study of these pathways becomes even more important, since it may generate relevant and transformative knowledge regarding the nature of cellular communication, as well as the molecular basis for gene expression regulation in both normal and cancer cells. Therefore, identifying the range of mutations and/or molecular changes occurring in these major signaling pathways and how they interact across pathways is crucial for understanding breast cancer development, as well as for implementation of molecular-based approaches for continuous improvement in diagnostics, prognostics, and treatment of breast cancer patients.

## Abbreviations

AF-1, N-terminal activation function 1 domain; AKT, Serine/threonine protein kinase 1; AMPK, AMP-activated protein kinase; AR, Androgen receptor; Areg, Amphiregulin; ARF, ADP ribosylation factor; ASPP2, Apoptosis-stimulating of p53 protein 2; ATP, Adenosine triphosphate; BAFF-R, B-cell activation factor; *BRCA1*, Breast cancer 1 suppressor gene; *BRCA2*, Breast cancer 2 suppressor gene; BTIC, Breast tumor initiating cell; CBP, CREB-binding protein; CDC42, Cell division cycle 42; COUP-TFI, Chicken ovalbumin upstream promoter-transcription factor-1; COX2, Cyclooxygenase-2; CSC, Cancer stem cell; CSF, Colony stimulating factor; *CYLD*, Cylindromatosis human gene; DAAM, Dvl-associated activator of morphogenesis; DKK1, Dickkopf-related protein 1; DMBA, 7,12-Dimethylbenz[a]anthracene; DNA, Deoxyribonucleic acid; dsDNA, Double-stranded DNA; Dvl, Dishevelled; E2, Estradiol; 17 $\beta$ -estradiol; EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; EMT, Epithelial-mesenchymal transition; ER, Estrogen receptor; ErbB2, Human epidermal growth factor receptor 2; ERE, Estrogen response element; ERK, Extracellular signal-regulated kinase; ERR $\alpha$ , Estrogen-related receptor alpha; ERR $\beta$ , Estrogen-related receptor beta; ERR $\gamma$ , Estrogen-related receptor gamma; FAK, Focal adhesion kinase; FERM, Four-point-one motif, ezrin, radixin, moesin; FN14, Fibroblast growth factor-inducible 14; FOXO, Forkhead box O; FZD, Frizzled receptor; GATA3, GATA binding protein 3; GH, Growth hormone; GM-CSF, Granulocyte macrophage colony-stimulating factor; GOF, Gain-of-function; GPCR, G protein-coupled receptor; GPER1, G protein-coupled ER 1;

GRB-2, Growth factor receptor-bound protein 2; GRB-7, Growth factor receptor-bound protein 7; GSK3 $\beta$ , Glycogen synthase kinase 3 $\beta$ , G $\beta\gamma$ , G beta-gamma complex; HDAC1, Histone deacetylase 1; HDM2, Human double minute 2 protein; IFN, Interferon; IGF1, Insulin-like growth factor 1; IKK, I $\kappa$ B kinase complex; IKK $\alpha$ , Inhibitor of nuclear factor kappa-B kinase subunit alpha; IKK $\beta$ , Inhibitor of nuclear factor kappa-B kinase subunit beta; IL-10, Interleukin 10; IL-1 $\beta$ , Interleukin 1 beta; IL-6, Interleukin 6; IRS-1, Insulin receptor substrate 1; I $\kappa$ B, Inhibitor of kappa B; JAK, Janus kinase; JNK, Jun amino-terminal kinase; LBD, Ligand-binding domain; LOF, Loss-of-function; LRP, LDL-related receptor protein; LRP5/6, Lipoprotein receptor-related protein; LT $\beta$ R, Lymphotoxin  $\beta$  receptor; MAPK, Mitogen-activated protein kinase; MAPKKK, Mitogen-activated protein kinases kinases kinase; MCF7/LCC1, Breast ductal carcinoma cell line; MEK, Mitogen-activated protein kinase kinase (or MAPKK, MKK); MMTV, Mouse mammary tumor virus; mTOR, Mammalian target of rapamycin; NF- $\kappa$ B, Nuclear factor kappa-light-chain-enhancer of activated B cell; NGFI-B, Nerve growth factor induced-B; NIK, NF- $\kappa$ B inducing kinase; NO, Nitric oxide; NOS-2, Nitric oxide synthase, inducible; NR, Nuclear receptor family; PAK1, P21 (RAC1) activated kinase 1; PCP, Planar cell polarity; PDK, Putative 3-phosphoinositide-dependent kinase (or PDK1); PGAP3, Post-GPI attachment to proteins 3; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PIK3CA, Phosphoinositide 3-kinase catalytic subunit  $\alpha$ ; PIP2, Phosphatidylinositol-4,5-bisphosphate; PIP3, Phosphatidylinositol-3,4,5-trisphosphate; PKB, Protein kinase B; PKC, Protein kinase C; PR, Progesterone receptor; proHB-EGF, Membrane-anchored heparin-binding EGF like growth factor; PTEN, Phosphatase and tensin homologue; PyMT, Polyomavirus middle T-antigen oncogene; RANK, Receptor activator for nuclear factor kappa B; RAPTOR, Regulatory-associated protein of mammalian target of rapamycin; RHR, Rel homology region; RIP1, Receptor Interacting Serine/Threonine Kinase 1; ROCK, Rho-associated kinase; ROR $\alpha$ , Retinoic acid-related orphan receptor alpha; RTK, Receptor tyrosine kinase; SC, stem cell; SERD, Selective estrogen receptor degrader; SERM, Selective estrogen receptor modulator; SH2, Src homology-2; SHC, Src Homology 2 domain-containing-transforming protein C1; siRNA, Small interfering RNA; SMAD, Contraction of SMA (small body size) and MAD (Mothers against decapentaplegic); SNAI2, SNAIL family transcriptional repressor 2; SOS, Son of sevenless; SRC, V-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog; STAT, Signal transducer and activator of transcription; TAD,

Transcription activation domain; TBK1, TANK-binding kinase 1; TCF/LEF, T-cell factor/lymphoid enhancer factor; TGF $\beta$ , Transforming growth factor  $\beta$ ; Th17, Helper T cells 17; TNBC, Triple-negative breast cancer; TNFR2, Tumor necrosis factor receptor 2; TP53, tumor protein 53 (or p53); TRAF, TNFR-associated factor; TSC, Tuberous sclerosis; TWEAK-R, TWEAK receptor; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; TYK2, Tyrosine kinase 2 or tyrosine-protein kinase 2; ULK1, Unc-51-like kinase 1; WNT, Wingless (wg)/integration 1 gene (*int*); ZEB1, Zinc finger E-box-binding homeobox 1; ZEB2, Zinc finger E-box-binding homeobox 2;  $\beta$ -TRCP, Beta-transducin repeat containing E3 ubiquitin protein ligase.

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