Review

Key signalling nodes in mammary gland development and cancer

Mitogen-activated protein kinase signalling in experimental models of breast cancer progression and in mammary gland development

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Abstract

Seven classes of mitogen-activated protein kinase (MAPK) intracellular signalling cascades exist, four of which are implicated in breast disease and function in mammary epithelial cells. These are the extracellular regulated kinase (ERK)1/2 pathway, the ERK5 pathway, the p38 pathway and the c-Jun N-terminal kinase (JNK) pathway. In some forms of human breast cancer and in many experimental models of breast cancer progression, signalling through the ERK1/2 pathway, in particular, has been implicated as being important. We review the influence of ERK1/2 activity on the organised three-dimensional association of mammary epithelial cells, and in models of breast cancer cell invasion. We assess the importance of epidermal growth factor receptor family signalling through ERK1/2 in models of breast cancer progression and the influence of ERK1/2 on its substrate, the oestrogen receptor, in this context. In parallel, we consider the importance of these MAPK-centred signalling cascades during the cycle of mammary gland development. Although less extensively studied, we highlight the instances of signalling through the p38, JNK and ERK5 pathways involved in breast cancer progression and mammary gland development.

Introduction

Four dominating mitogen-activated protein kinase (MAPK) signalling cascades are implicated in breast disease in mammalian cells: the extracellular regulated kinase (ERK)1/2 pathway, the c-Jun N-terminal kinase (JNK) pathway, the p38 pathway and the ERK5 pathway [1,2]. In some forms of human breast cancer and in many experimental models of breast cancer progression, signalling through the ERK1/2 pathway, in particular, has been implicated as being important [3]. Signalling through each pathway involves sequential activation of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and the MAPK. Considering the ERK1/2 pathway, the primary input activator is activated Ras, a small GTPase. It activates Raf1 (MAPKKK), which then phosphorylates and activates MEK1/2 (MAPKK), which finally activates ERK1/2 [1]. In its active form, ERK1/2 phosphorylates a wide range of protein substrates (on serine or threonine). MAPKKKs and MAPKKs are dual Thr/Tyr kinases. Signalling through the core to activate the MAPK is supported on a protein scaffold [4]. However, activated ERK1/2 is translocated from the scaffold to, for example, the nucleus, where it phosphorylates its substrates. The activity of the core Raf1-MEK1/2-ERK1/2 cascade is auto-regulated by ERK1/2 inactivation of MEK1 or by ERK1/2 upregulation of ERK1/2-specific phosphatases, for example, MKP2 and 3 (MAPK dual-specificity phosphatases) [5,6]. In this way, ERK1/2 itself can exert control over the intensity and duration of ERK1/2 signalling.

The ERK1/2 cascade is triggered by growth factors and cytokines acting through receptor tyrosine kinases, G-protein-coupled receptors, and non-nuclear activated steroid hormone receptors [4,7-13]. ERK1/2 substrates include transcriptional regulators, apoptosis regulators and steroid hormone receptors (for example, oestrogen receptor (ER)α). The biological consequences of ERK1/2 substrate phosphory-

AP-1 = Activator protein-1; aPKC = atypical PKC; BH3 = Bcl-2 homology domain 3; ECM = extracellular matrix; EGF = epidermal growth factor; EGFR = EGF receptor; EMT = epithelial-to-mesenchymal transition; ER = oestrogen receptor; ERK = extracellular regulated kinase; FGF = fibroblast growth factor; IGF = insulin-like growth factor; IGF-1R = IGF receptor; IL = interleukin; JNK = c-Jun N-terminal kinase; MAPK = mitogen-activated protein kinase; MAPKK = MAPK kinase; MAPKKK = MAPK kinase kinase; MEC = mammary epithelial cell; MMP = matrix-metalloproteinase; MMTV = mouse mammary tumour virus; Par = Partitioning defect; PI3K = phosphoinositide 3-kinase; PKC = protein kinase C; TGF = transforming growth factor; uPA = Plasminogen activator, Urokinase; uPAR = Urokinase receptor; VEGF = vascular epidermal growth factor.

lation include pro-proliferative [14], pro-differentiation [14], pro-survival [14], pro-angiogenic [15], pro-motility [16] and pro-invasive effects [17]. We review the contribution of MAPK signalling events to breast cancer progression as reflected in experimental models of progression (contrasting this to their role in normal mammary gland development).

ERK1/2 signalling in models of breast cancer progression

In approximately 30% of human breast cancers, mutations are found in the ERK1/2 MAPK pathway; however, the nature of the deregulation varies between individual tumours and cancer subtypes [18,19]. Dys-regulated ERK1/2 signalling alone is usually not sufficient to cause cancer. Additional mutations and changes in the expression levels of other genes is commonly observed alongside (for example, those encoding ErbB2 receptor, c-myc, p53 and hyaluronan-mediated motility receptor (RHAMM)) [20,21]. Furthermore, other major signalling networks (such as phosphoinositide 3-kinase (PI3K)/phosphatase and tensin homolog (PTEN)/AKT) crosstalk with ERK1/2 signalling, thereby enhancing cell proliferation, preventing apoptosis and, in some cases, inducing drug resistance [22]. ERK1/2 is hyper-activated in a large subset of mammary tumours, and downstream ERK1/2 targets are also hyper-phosphorylated there [23]. There is a trend for higher ERK1/2 activity in primary tumours of 'node-positive' patients than in those from 'node-negative' patients, suggesting higher ERK1/2 activity impacts metastasis [23]. A survival analysis demonstrated that low ERK1/2 activity in primary breast tumours is prognostic for relapse-free survival of patients [23]. The expression and activity of ERK1/2 can also impact a patient's response to treatment [3,24]. Over-expression of upstream ERK1/2 modulators (such as MEK1) in normal mammary epithelial cells induces neoplastic transformation, highlighting the relevance of this signalling network to breast cancer [25]. Although balanced against the above are a series of clinical studies in three cohorts of patients that link high ERK1/2 and/or high activated (phosphorylated) ERK1/2 levels to good disease outcome [26-28]; the apparent contradictory nature of the two streams of data remains to be resolved.

Immortalized mammary epithelial cell lines with normal phenotype (including MCF10A/12A, S1, NMuMG, epH4, human mammary epithelial cell and HB2), breast cancer cell lines (including MCF-7, T47D, MDA-MB-231, T-42, SKBR-3, BT549) and primary mammary epithelial cells (of both normal and tumour origin) can be cultured and are commonly used to model the molecular contribution of MAPK signalling in normal epithelia and in breast cancer. We describe a range of such studies. In addition, we present a summary in Table 1.

Sustained ERK1/2 signalling undermines epithelial cell organisation

Primary cells and various phenotypically normal mammary epithelial cell lines, when supported on a reconstituted basement membrane (extracellular matrix (ECM)) mimic *in vivo* alveolus cell organisation and form three-dimensional spherical acini. Firstly, the outer layer of cells in contact with the ECM polarise and exit the cell cycle. Lumen clearance then occurs (at least in part by apoptosis) to generate the acinus in which the mammary epithelial cells (MECs) are organised in organotypic fashion [29]. Of particular interest is that transformed MECs do not organise in this fashion and their failure to do so is often determined by persistent ERK1/2 signalling (reviewed in [30]). For instance, tumourigenic HMT-3522 T-42 MECs fail to organise as acini because of a persistent β1-integrin-epidermal growth factor receptor (EGFR)-ERK1/2 drive. However, they 'revert' and form acini (like their isogenic 'normal' S-1 cell counterparts) if β1-integrin, EGFR or ERK1/2 function is inhibited [31]. Importantly, this bidirectional cross-modulation of β1-integrin and EGFR signalling via the MAPK pathway is dependent upon a three-dimensional context. Wang and colleagues [31] showed how partial 'reversion' in functional phenotype of a range of breast cancer cells could be achieved by inhibiting ERK1/2. A more complete 'reversion' was possible with multiple signalling inhibitors (for example, of ERK1/2 plus PI3K pathways). Thus, persistent ERK1/2 signalling contributes to, but need not necessarily be the only, intracellular signalling change that is driving loss of epithelial characteristics in breast cancer cells. Finally, in breast cancer SKBR-3 cells grown in monolayer culture, ErbB2 and ErbB3 form heterodimers, whereas in multicellular spheroids ErbB2 homodimers are formed, leading to enhanced activation of ErbB2 and a switch in signalling pathways from PI3K to ERK1/2 [32]. Thus, comparisons of three-dimensional and monolayer cultures emphasise the use of three-dimensional systems to better reflect some *in vivo* aspects of ErbB and ERK1/2 signalling.

Expression of an oncogene was found to be sufficient to impair acinus formation by normal MECs while in parallel triggering activation of ERK1/2 signalling [33]. Persistent activation of fibroblast frowth factor (FGF) receptor in HC11 MECs caused ERK1/2 activation, impaired acinus formation and led to an invasive phenotype [34]. Likewise, persistent activation of ErbB2 in MCF-10A cells, which is associated with activation of ERK1/2, caused aberrant acinus maturation but the cells were not invasive [35]. However, addition of transforming growth factor (TGF)β1 induced a migratory and invasive behaviour in these cells. Significantly, blocking ERK1/2 activation inhibited the effect of TGFβ1 and ErbB2 on migration.

Nolan and colleagues [36] show that, in three-dimensional assemblies, activated ErbB2 recruits the cell polarity regulators Partitioning defect (Par)6-atypical protein kinase C (aPKC) from their association with Par3. This disrupted cell polarisation and caused luminal cell survival (but not additional cell proliferation) and the formation of dysmorphic, multi-acinar spheres. In contrast, if Par6 is over-expressed, it

acts in a complex with cdc42 and aPKC to cause hyper-proliferation and generate multi-acinar structures in an ERK1/2-dependent fashion [36]. Thus, disrupting the cell polarisation machinery can affect control of cell proliferation and/or cell survival and so impact lumen clearance.

The Bcl-2 homology domain (BH3)-only pro-apoptotic proteins Bim and Bmf (Bcl-2 modifying factor) selectively trigger apoptosis of central cells, leading to lumen formation in the acinus [37,38]. Oncogenes that drive luminal filling, such as ErbB2, suppress Bim expression and drive Bim inactivation by phosphorylation in an ERK1/2-dependent manner. Activation of the ERK1/2 pathway is sufficient to block Bim activity and correlates with protection from luminal apoptosis and inhibition of lumen formation [37]. In a parallel fashion, in pubertal mammary glands of Bim-/- mice, terminal end buds and ducts have filled lumena, presumably due to a deficit in pro-apoptotic signalling [39]. Thus, there is ample evidence supporting the identification of the ERK1/2 MAPK node as a signalling driver that must be tightly controlled if mammary epithelial cells are to successfully organise, organotypically.

Invasion - a role for ERK1/2 signalling

Metastatic breast tumours represent the final lethal stage of the disease, with cancer cells from the primary mammary gland site having 'spread' to a secondary site (for example, bone, lung or liver). 'Invasive' cells with de-regulated growth properties and an acquired ability to degrade basement membrane invade the local tissue environment, eventually reaching and circulating through the lymph and vascular systems before entering other tissues and forming secondary tumours. Epithelial cells that have undergone an epithelial-to-mesenchymal transition (EMT) display many of the characteristics of invasive cancer cells, in that they have lost their adhesions and polarised phenotype, have down-regulated epithelial-specific proteins (such as tight junction components) and have gained a proliferative and migratory/invasive phenotype with an ability to degrade and migrate through ECM. A range of growth factors and cytokines (for example, human growth factor, FGF and TGFβ) have been found to trigger EMT [40-45]. But in particular, cooperation between TGFβ and sustained ERK1/2 signalling induces EMT and an invasive and metastatic potential in cultured mammary epithelial cells [40]. TGFβ signals through transmembrane receptor kinases and activates p38 and ERK1/2 through both Smad-dependent and Smad-independent mechanisms and the MAPK4 pathway [46,47]. TGFβ inhibits growth of normal epithelial cells but induces proliferation and EMT in immortalised, phenotypically normal MCF10A cells and in cells from advanced carcinomas [48].

During normal mammary gland development, TGFβ isoforms seem required for pubertal ductal morphogenesis [49], pregnancy-induced alveolar morphogenesis (in part by mediating the action of progesterone) [49,50], for normal transition from pregnancy to lactation and to drive involution [51]. In pubertal mammary gland development, TGFβ inhibits ductal growth and its role may be in sculpting the newly developing mammary tree: branches form at sites of lowest local TGFβ concentration [52]. Little is known of intracellular signalling networks that support ductal branching; however, in a pubertal mammary explant culture it was shown that sustained ERK1/2 activation induced by TGFα caused branching, while transient ERK1/2 activation induced by FGF7 did not. It was hypothesised that the former would trigger necessary matrix-metalloproteinase (MMP) expression and the latter not [7].

Invasive cells synthesise MMPs, which degrade type IV collagen (and other matrix components) and confer on cells the ability to migrate and invade neighbouring tissue. ERK1/2 signalling induces MMP expression/activity, thereby impacting extracellular re-modelling [53]. In breast cancer cell cultures, invasiveness and MMP2 and MMP9 activity are induced in response to TGFβ stimulation and ErbB2 overexpression [54,55] and requires p38 activity in some circumstances. The serine protease uPA (Plasminogen activator, Urokinase) and its receptor (uPAR) also play a crucial role in breast cancer cell invasion and metastasis alongside MMPs. uPA induces cell proliferation in MDA-MB-231 breast cancer cells via ERK1/2 activation and binding of uPA to uPAR activates ERK1/2 in MCF-7 cells [8,56]. Endogenously produced uPA was found to be a major determinant of the basal level of activated ERK1/2 and prevented apoptosis in metastatic MDA-MB-231 cells [57]. ERK1/2 signalling in MDA-MB-231 breast carcinoma cells is adapted to obtain high urokinase expression and rapid cell proliferation; there, inhibition of ERK1/2 activity leads to decreased cell proliferation and decreased cyclin D1 expression [58]. In the normal mammary gland, MMPs orchestrate extensive reductive re-modelling in the later phase of involution via degradation of ECM, and MMP2, 3, 7, 9 and 11 are important to this processing [59], which is probably driven by cytokine-triggered ERK1/2 activity (see below) [60,61].

ERK1/2 activity has been highlighted in several studies of invasion (reviewed in [62]). Cell-cell adhesion molecules (including E-cadherins and tight junction proteins such as ZO-1 (zona occludens 1)) play a pivotal role in maintaining normal breast epithelial architecture. A hallmark of EMT is the loss of E-cadherin expression and an increase in the expression of N-cadherin and vimentin. EGF-induced EGFR activation destabilises E-cadherin adhesions [63]. However, it is known from several studies that re-expression of E-cadherin alone may not be sufficient to restore regulated cell growth and epithelial-like behaviour. For example, in MCF10A cells over-expressing activated Ras, restoration of an epithelial cell phenotype required both the over-expression of E-cadherin and the suppression of ERK1/2 activity [64]. Overexpression of N-cadherin in MCF-7 cells induces cell migration *in vitro* and invasion and metastasis *in vivo* by an ERK1/2-dependent mechanism [65,66].

Table 1

Cell culture based experimental characterisation of ERK1/2 association with breast cancer pregression

	Model	Reference
ERK1/2 signalling		
MEK1 signalling mediates transformation and metastasis	EpH4 mammary epithelial cells	[25]
RAF/MEK/ERK1/2 and PI3K/PTEN/AKT signalling pathways interact in breast cancer	Hematopoietic, breast (MCF7) and prostate cancer cells	[22]
Three-dimensional organisation		
MECs fail to organise as acini because of a persistent β1-integrin-EGFR-ERK1/2 drive, but will form acini if β1-integrin, EGFR or ERK1/2 function is inhibited	HMT-3522 T-42	[75]
Persistent activation of ERK1/2 impairs acinus formation and leads to invasion	HC11 MECs	[34]
Delayed activation of ERK1/2 impacts cell proliferation and ERα-mediated transcription	MCF7	[82]
Over-expressed Par6 acts in a complex with cdc42 and aPKC to induce hyperproliferation and generate multi-acinar structures in an ERK1/2-dependent fashion	MECs	[36]
Activation of the ERK1/2 blocks Bim expression and correlates with protection from luminal apoptosis	MECs	[37]
Invasion		
Ha-Ras cooperates with TGFβ to induce EMT and Raf/ERK1/2 is required	Ha-Ras-transformed MECs in 3D collagen/matrigel matrices	[40]
ERK1/2 signalling induces MMP expression and the duration of MAPK activation is an important determinant for certain growth factor-mediated functions	Keratinocytes	[53]
uPA binding to uPAR activates ERK1/2 and induces cell migration	MCF7	[8]
uPA induces cell proliferation via ERK1/2 activation	MDA-MB-231	[56]
uPA determines the basal level of activated ERK1/2 and prevents apoptosis	MDA-MB-231	[57]
Restoration of an epithelial phenotype requires both the over-expression of E-cadherin and the suppression of ERK1/2	MCF10A cells over-expressing activated Ras	[64]
Scribble co-operates with mutations in Ras and Raf to induce a migratory phenotype via induction of ERK1/2	MCF10A	[67]
ECM changes impact integrin signalling and can promote mitogenic signalling through ERK1/2	Non-malignant and human breast tumour cell line (T4-2)	[75]
ERK1/2 substrates, the Ets transcription factors, induce EMT and invasiveness	MECs	[76-78]
'Tumour-initiating cells' can be derived from mammary cells following the activation of ERK1/2 and induction of EMT	MECs	[81]
ErbB/EGFR signalling to ERK1/2		
Overexpression of ErbB2 induces EMT through ERK1/2 activation	MCF10A	[90]
Expression of ErbB2 induces anti-apoptotic proteins Survivin and Bcl-2 via ERK1/2 and PI3K signalling	MCF7	[87]
Experimentally triggered ErbB2 activation protects against apoptosis and disrupts mammary epithelial cell organisation in an ERK1/2-dependent manner	MCF10A	[88,89]
Progesterone receptor, IGF-1, VEGF, growth hormone and a range of ligands require EGFR to induce ERK1/2 activation	T47D, MECs	[91]
ER, tamoxifen resistance and ERK1/2 signalling		
ERK1 and 2 are activated via oestrogen signalling through GPR30, resulting in transactivation of EGFR	MCF7, SKBR3 breast cancer cells	[10]
EGFR or ErbB2 resistance correlated with high ERK1/2 and AKT activity	Breast cancer cells	[9]
Cell survival and cell death		
Survival factor-induced ERK1/2 phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity	Haematopoietic cells	[134]
ERK1/2 phosphorylates the pro-apoptotic BCL-2 family member Bim_{EL}, leading to its degradation by the proteasome	CC139 fibroblasts	[132,133]

aPKC = atypical PKC; EGF = epidermal growth factor; EGFR = EGF receptor; EMT = epithelial-to-mesenchymal transition; ER = oestrogen receptor; ERK = extracellular regulated kinase; IGF = insulin-like growth factor; MAPK = mitogen-activated protein kinase; MEC = mammary epithelial cell; MMP = matrix-metalloproteinase; Par = Partitioning defect; PI3K = phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; TGF = transforming growth factor; uPA = Plasminogen activator, Urokinase; uPAR = Urokinase receptor; VEGF = vascular epidermal growth factor.

Polarity proteins (such as Par-, Crumbs- and Scribble-complex proteins) determine the establishment and maintenance of cell polarity in epithelial cells (as reflected, for instance, by the asymmetric distribution of cadherin and tight junctions). But they have been shown to redeploy and co-operate in a sustained ERK1/2 signalling environment to induce abnormal cell growth and invasive potential [36]. And, suppressed expression of the polarity regulator Scribble in MCF10A cells was found to co-operate with mutations in Ras and Raf to induce a migratory phenotype via induction of ERK1/2 (but not p38 or JNK) signalling [67].

Alongside a loss of cell-cell adhesions and polarity, cytoskeleton re-modelling and the formation of cellular protrusions are a feature of breast cancer cell migration and early metastasis. The dynamics and regulation of the actin cytoskeleton is central to migratory processes and may be corrupted at several levels in invasive cancers (reviewed in [68]). Induced ERK1/2 signalling can inactivate integrin-mediated cell adhesion and activate myosin light chain kinase, thereby impacting cell migration [69]. Also central to migratory processes are focal adhesion components, for instance Focal adhesion kinase, which is activated by integrins and by growth factor receptors, including the EGFR family [70]. Focal adhesion kinase is hyper-activated during the early stages of invasion responses in cultured breast cancer cells and was found to both stimulate cell proliferation [60] and inhibit apoptosis [71]. It is also hyper-activated in ErbB2-positive breast cancers [72]. Interestingly, mice with conditional Focal adhesion kinase knockout in the mammary gland display decreased alveolar epithelial cell proliferative capacity during late pregnancy, with reduced cyclin D1 expression and reduced ERK1/2 phosphorylation [73].

Another environmental cue, tissue rigidity (due to a stiffer ECM), can promote tumour progression (reviewed in [74]). ECM changes impact integrin signalling and can promote mitogenic signalling through EGFR-ERK1/2 signalling and also cell contractility through Rho, which can further increase matrix stiffness [75], contributing to the cycle of tumour growth and progression.

Key ERK1/2 substrates, the Ets-1 transcription factors, have been shown to induce EMT and invasiveness in a series of human breast cancer cell lines. The Ets-factor-ESE-1 transforms MCF-12A mammary cells in culture [76]. Ets-1 activity promotes breast cancer cell metastasis (and ERK1/2 signalling) by driving the expression of MMPs, FGF, uPA, uPAR and EGF [77,78].

'Cancer stem cells' are potentially rare cells with both stem and tumourigenic properties. They have an indefinite potential for self-renewal and are hypothesised to drive tumourigenesis [79]. The existence of a subpopulation of breast cancer stem cells was demonstrated recently when they were isolated from breast cancer pleural effusions by limiting dilution transplantation of the cells into the mammary fat pad of immuno-compromised mice [80]. 'Tumour-initiating cells' can be derived from cultured mammary cells following the activation of the ERK1/2 pathway and induction of EMT and such cells are found in ErbB2-induced mammary tumours [81,82]. While the capability of stimuli that drive EMT to enhance the abundance of breast cancer stem cells is most interesting and will provide a ready experimental model in which to study their generation, the relevance of the phenomenon to breast tumour progression remains to be established.

ErbB/EGFR family signalling to ERK1/2

Of the growth factor receptors that input signals to ERK1/2 in breast tumour cells, the ErbB/EGFR family of receptor tyrosine kinases is one of the major classes: they are commonly over-expressed (for example, ErbB2 gene amplification occurs in 20 to 30% of tumours [19]) or persistently signal (for example, EGFR in tamoxifen resistance [83]) in human breast tumours. An anti-receptor antibody, trastuzumab, is used with some success in treating disease with amplified ErbB2; gefitinib, an EGFR kinase inhibitor, is used to treat patients exhibiting tamoxifen resistance [84]. A family of ten ligands, including EGF, amphiregulin and heregulin, bind the ErbB receptors, induce receptor dimerization, trigger kinase activity, autophosphorylation and downstream activation of ERK1/2 and PI3K-AKT signalling. There are four closely related ErbB receptors (ErbB1/EGFR, ErbB2/Her-2/Neu, ErbB3 and ErbB4) that form hetero- or homo-dimers. ErbB receptor signalling can increase cell proliferation, decrease apoptosis and affect the survival and motility of primary and metastatic breast tumour cells. ErbB2 action also enhances signalling interactions with the cellular micro-environment and affects cell adhesion (reviewed in [85]).

The potential contribution of the ErbB receptor-ERK1/2 signalling has been widely studied in experimental model systems of progression (reviewed in [19]). In a range of breast cancer cell lines hyper-proliferation is mediated by EGFR- or ErbB2-dependent ERK1/2 activation (reviewed in [86]). ErbB2-ERK1/2 signalling may affect breast cancer cell behaviour through influencing cell survival as much as through affecting proliferation: expression of ErbB2 in MCF-7 cells induces the anti-apoptotic proteins Survivin and Bcl-2 via ERK1/2 and PI3K signalling [87], and experimentally triggered ErbB2 activation protects against apoptosis and disrupts MCF10A epithelial cell three-dimensional organisation in an ERK1/2-dependent manner [88,89]. Over-expression of ErbB2 induces EMT through ERK1/2 activation in MCF10A human breast epithelial cells [90]. While EGFR-ERK1/2 signalling is conventionally ligand-activated, this may often arise through an autocrine mechanism; MMP9 partially acts to drive invasiveness through induction of EGF expression [91]. Interestingly, in MCF10A and a range of breast cancer cell lines, activation or over-expression of ErbB2 has been shown to increase cell growth *in vitro*, to

initiate tumours *in vivo*, to activate p38 and to induce cell migration via up-regulation of MMP-9 (which may involve autocrine EGFR activation). Common also is activation of this signalling by EGFR trans-activation, which has been shown to be induced by G-protein-coupled receptors [92,93]. Furthermore, in a single cell line insulin-like growth factor (IGF)-1, vascular epidermal growth factor (VEGF), growth hormone and a range of ligands all require EGFR to induce ERK1/2 activation [91]. In addition, the progesterone receptor was recently found to upregulate WNT-1 and to induce EGFR transactivation and a sustained activation of ERK1/2 in T47D cells growing in an adhesion-independent fashion in soft agar [94].

Promoters that selectively drive gene expression in the mammary gland (that is, those for mouse mammary tumour virus (MMTV) and whey acidic protein) have allowed the creation of useful mouse models for studying breast cancer *in vivo*. The best characterised models are those expressing the viral oncogene encoding the polyoma middle T antigen (MMTV-PyV MT) as paradigm of viral transforming potential, and that expressing ErbB2 (MMTV-Neu) [95,96]. A complementation study that crossed tyrosine phosphatase PTP1B knockout mice into this ErbB2 over-expression background led to down-regulation of ERK1/2 signalling and delayed tumour development, suggesting a role for the ERK1/2 pathway in ErbB2-induced mammary tumourigenesis [97,98]. A direct role for ERK1/2 in induction of mammary tumour progression has been recently shown *in vivo*, using cells transplanted into the mammary fat pad [99]. The authors showed that ERK1/2 directly modulates the phosphorylation and degradation of the tumour suppressor FOXO3a (Forkhead box 3), leading to a decrease in the anti-tumourigenic activity of this protein in the mammary gland. Lastly, the EphA2 receptor tyrosine kinase, a regulator of mammary gland branching morphogenesis [100], which drives Ras-ERK1/2 and RhoA GTPase signalling, has been shown to enhance tumour formation in MMTV-Neu mice, but not in MMTV-PyV MT mice [101], again potentially linking the ERK1/2 pathway to ErbB2-induced tumourigenesis.

Thus, in breast cancer, EGFR/ErbB receptors are commonly over-expressed [19] or persistently signal (in tamoxifen resistance [83]), and an anti-receptor antibody is being used with success in treating disease with amplified ErbB2, and an EGFR kinase inhibitor is being used to treat tamoxifen-resistant disease [84]. In cell culture-based experimental model systems it is clear, as outlined above, that a functional link can be made between EGFR/ErbB receptor activation, induction of ERK1/2 pathway signalling and relevant outcomes such as increased proliferation [102], cell survival [103] and induction of motility [16] and invasiveness [17]. But does this qualify the MEK1-ERK1/2 signalling pathway as a useful therapeutic target in breast cancer, generally? Available clinical evidence linking high activated ERK1/2 levels to a good disease outcome [26-28] would say no. But, for instance, in a subset of cases where anti-EGFR therapy fails there may be some potential [84]. But perhaps the most useful experimental endeavour would be to develop additional cell-based models to more accurately mimic progression of particular disease subtypes in order to define the dominant supporting intracellular signalling pathways and, thus, new key therapeutic targets.

Oestrogen receptor, tamoxifen resistance and ERK1/2 signalling

The biological actions of oestrogen are mediated both by transcriptional regulation (involving ERα and ERβ in the nucleus) and by non-genomic actions via ligand binding to ERα at the plasma membrane [104-106]. In addition, ERK1/2 phosphorylates ERα on sites in its amino-terminal ligand-independent transcriptional activating domain (Ser118 and Ser167) [83,107]. Ser167 is phosphorylated by AKT, p90 Ribosomal S6 kinase, and casein kinase II as well as by ERK1/2 [108]. Phosphorylation on Ser118 by ERK1/2 increases the transcriptional activation function, thus providing a means by which ERα can be activated in a ligand-independent manner through growth factor receptor signalling. It may also facilitate blocking of inhibitory actions of anti-oestrogens/selective ER modulators on ERα in breast cancer cells and, hence, contribute to tamoxifen resistance; the phosphorylation event may act to reduce the binding of the high affinity ligand, 4-hydroxy-tamoxifen, to ERα [83]. It has been shown that ErbB2-over-expressing MCF-7 cells are tamoxifen resistant; they have high levels of activated ERK1/2 and high levels of phosphorylation of ERα at Ser118 [109]. MEK inhibitors reverse this resistance [110]. But interestingly, several studies have linked elevated levels of phosphorylation at Ser118 of ERα to a positive survival outcome for breast cancer patients [26,111]. One study positively correlates elevated levels of ERK1/2 activation with elevated phosphorylation of ERα at Ser118 and with a good survival outcome [26]; here also phosphorylation of ERα at Ser118 inversely correlated with ErbB2 expression, suggesting that ErbB2 is not primarily signalling through ERK1/2 in this context [83]. The relationship of phosphorylation at the other ERK1/2 target, Ser167, to outcome for patients and to tamoxifen resistance is less than clear; suffice it to say that AKT rather than ERK1/2 would seem to be its major generator [83].

ERK1 and ERK2 are also activated via oestrogen signalling through the G-protein-coupled receptor GPR30, which results in transactivation of EGFR in MCF-7 cells and in SKBR3 breast cancer cells [10]. However, GPR30 may play a broader role in balancing ERK1/2 activation as oestrogen signalling via the G-protein-coupled receptor inhibits ERK1/2 activity by a cAMP-dependent mechanism, which results in Raf-1 inactivation in breast carcinoma cells [112]. It is implied that oestrogen may drive cellular responses that are growth factor dependent via GPR30 in ER-negative breast tumours [10].

Splice isoforms of ER exist that account for the heterogeneity of ER gene expression, which is also tissue-specific [113,114]. Post-translational modification by palmitoylation recruits an abundant amino-terminally truncated ERα (46 kDa) to the plasma membrane in human endothelial cells, osteoblasts and MCF-7 cells [113,115,116]. ER46 mediates rapid membrane-initiated oestrogen responses, including activation of endothelial nitric oxide synthetase [116,117]. A functional signalling node composed of ER46, c-Src and p85 (the regulatory subunit of PI3K) has been identified in the oestrogen-induced activation of endothelial cells, and is directed by c-Src [118]. The importance of the presence of membrane associated isoforms of ER lie in their ability to illicit rapid and non-transcriptional responses to oestrogen [114].

Anti-hormone treatment with selective ER modulators, most commonly tamoxifen, act to inhibit ER activity in breast cancer cells and their metastases; aromatase inhibitors block oestrogen biosynthesis and reduce endogenous ligand-dependent ER activation [83,119]. In suitable patient cohorts both therapies prolong survival, but resistance to these therapeutics limits their use [83]. In cell culture studies in which EGFR or ErbB2 have been over-expressed and anti-oestrogen resistance induced [120], or resistance induced by prolonged exposure to anti-oestrogens [9,121], resistance correlated with high ERK1/2 and AKT activity. There is accumulating evidence that growth factor-triggered ERK1/2 signalling contributes significantly to tamoxifen resistance but it may do so by multiple mechanisms. Firstly, both tamoxifen-resistant tumours and cell lines tend to be rich in EGFR and ErbB2 receptors and their activation of ERK1/2 and PI3K-AKT would have significant anti-apoptotic effects. Secondly, ERK1/2 phosphorylation of Ser118 in ERα may also contribute to resistance [83]. And finally, non-nuclear ER activity may contribute: the IGF receptor (IGF-1R) acts upstream of ErbB1 in a signalling pathway in MCF-7 cells, which mediates oestrogen activation of ERK1/2, with subsequent cell growth stimulation and protection against apoptosis. Oestrogen stimulated the phosphorylation of both IGF-1R and ErbB1, and inhibitor studies that blocked this pathway also blocked ERK1/2 activation and the mitogenic and anti-apoptotic effects of oestrogen [9].

The tamoxifen resistance phenotype is predominantly associated with tumour re-growth rather than metastasis [83]. However, Santen and colleagues [3] comment that the phenotype (potentially sustained by EGFR/ErbB2-ERK1/2/PI3K-AKT signalling) is that that would promote invasion and metastasis. Interestingly, when over-expressing Ras mutants in NIH 3T3 fibroblasts to activate downstream effectors (Raf-ERK1/2, PI3K or RalGEF) and, thereby, tumour growth in nude mice, it was found that only the mutant that activated ERK1/2 (V12S35 Ras expressed in NIH 3T3 fibroblasts) also induced lung metastasis [122], highlighting the potential importance of sustained ERK1/2 signalling in metastasis.

Pubertal mammary gland ductal morphogenesis in the mouse requires epithelial cell ERα-induced EGFR ligand (amphiregulin) expression, as indicated by knockout/mammary fat pad transplantation studies [123]. Additional knockout/transplantation studies indicated that the target for the amphiregulin was stromal cell EGFR, without which the phased epithelial proliferation and branching of mammary tree development fails [124]. This relationship and its morphological outcome contrasts with the ER-EGFR-family-ERK1/2 signalling interactions seen in 'ErbB2 amplified' breast tumours and tamoxifen-resistant disease, where we suppose a dominant and sustained ERK1/2 signalling contributes. Additional studies have demonstrated the presence of an ER-controlled Src-ERK1/2 phosphorylation cascade functioning during pubertal ductal morphogenesis [125].

Cell survival and cell death

Both ERK1/2 activation and its inhibition can induce apoptosis, with the output dependent on the cellular context and input signalling. ERK1/2 plays a role in apoptosis induced by a range of therapeutics in breast cancer cells. ERK1/2 and p38 signalling was found to be required for resveratrol-induced apoptosis in MDA-MB-231 breast cancer cells [126,127] and activation of ERK1/2 and JNK, but not p38, was found to be required for vitamin E succinate-induced apoptosis of MDA-MB-435 breast cancer cells [128]. Genistein, Oligonol, kaempferol, and linoleic acid all induce apoptosis in breast cancer cells in an ERK1/2-dependant manner [129-131].

ERK1/2 phosphorylates the pro-apoptotic BCL-2 family member Bim_{EL} (a BH3-only domain protein) [132], leading to its degradation by the proteasome [133]. Bim initiates apoptosis, but after survival factor-induced ERK1/2-phosphorylation, Bim's association with Bax is inhibited, as is its pro-apoptotic activity, leading to cell survival [134]. As outlined earlier, Bim drives cell death during lumen clearance in the ductal terminal end buds of the developing pubertal mammary tree and during acinus formation [37,39]. But importantly, it drives the cell death of epithelial anoikis, whose inhibition is permissive for invasion and metastasis [135]. ERK1/2 action, regulated by EGFR or coordinately by EGFR and β1-integrin, negatively regulates Bim activity both at a transcriptional level and by phosphorylation; the former may be the principal mode of regulation [37]. The related BH3-only domain protein Bmf is expressed during involution, and is active in epithelial cell anoikis (monolayer culture) and in apoptosis/lumen clearance during acinus formation. Sustained ERK1/2 or PI3K activation suppresses this Bmf up-regulation, suggesting that, in this instance, active ERK1/2 was pro-survival [38]. Growth and survival factor-activated ERK1/2 signalling can also regulate Hdm2 expression (a negative regulator of p53), contributing to a pro-survival effect [136]. p53 itself can activate ERK1/2 signalling via transcriptional activation of the EGF gene [137]. Expression of p53 in other circumstances can reduce phospho-ERK1/2

levels and this inhibition of ERK1/2 signalling accompanies apoptosis inducing factor-mediated apoptosis in MCF-7 cells in response to metals [138]. The authors also found that suppression of p53 in MDA-MB231 and MCF7-E6 cells resulted in strong ERK1/2 phosphorylation.

Normal mammary gland involution is characterized by extensive alveolar epithelial cell apoptosis in its initial stages, and later by matrix degradation and tissue re-modelling directed by matrix-degrading MMPs 2, 3, 7 and 11, the plasminogen activation pathway and cathepsins (reviewed in [59]). In conditional Stat3-/- mice involution is delayed [139]. Stat3 is thus pro-apoptotic. IL6 expression increases with the onset of involution, and parallels the activation of Stat3 and ERK1/2 [61,140]. Leukemia inhibitory factor also mediates ERK1/2 activation at this time; however, sustained activation of ERK1/2 through to late involution requires Oncostatin M. It is suggested that the controlled re-modelling of mammary tissue at involution is regulated by IL-6, leukemia inhibitory factor and Oncostatin M, acting via ERK1/2 and by driving expression of pro-apoptotic Bax [61].

p38, JNK and ERK5

p38

p38 MAPK plays roles in cell differentiation, growth inhibition and apoptosis [141,142]. p38 MAPK is activated in cells in response to stress signals, growth factors, inflammatory cytokines, UV, heat and osmotic shock [142]. Its activation often leads to cell death. In addition, a new role for p38 in 'checkpoint control' during cell division and cell survival is now recognized [143]. Four isoforms of p38 exist (p38α, β, γ and δ), although p38α is the most widely expressed. MKK3/6 (MAPKKK) and SEK (MAPKK) activate p38. p38 is also activated via interactions with the scaffolding protein TAB1 [144]. Activated p38 phosphorylates and activates many transcription factors (including Activating transcription factor-2, Max, Myocyte enhancer factor-2, Mac, p53 and Stat1) [145,146].

Links between p38 and invasiveness in cultured cells have been reported: phospho-p38 is elevated in cultured invasive breast cancer cells and constitutive p38 activity induces the overproduction of the pro-invasive uPA [147]. Also, treatment of the invasive BT549 breast cancer cells with a p38 MAPK inhibitor diminished both uPA and uPAR expression and inhibited the ability of these cells to invade matrigel [147,148]. Furthermore, activated Src over-expression was shown to necessarily activate p38 during TGFβ-induced breast cancer cell proliferation and invasion [149]. Studies on mammary cells indicate that WAVE-3 (a regulator of cytoskeletal dynamics and cell motility) regulates breast cancer progression, invasion and metastasis through the p38 pathway and MMP production. Knockdown of WAVE3 using small interfering RNA in MDA-MB-231 cells decreases p38 activity but not AKT, ERK1/2 or JNK [150]. Elevated phospho-p38 levels have been associated with high expression of EGFR and ErbB2 as well in tamoxifen-resistant xenografts [151]. p38 would not be seen as driving apoptosis in this context; maybe it acts to support nuclear functions of ER [152]. And interestingly, a relationship between p38 and phospho-p38 levels and lymph node metastasis was identified in human breast cancer samples [151]. Most interestingly, in a study of matched primary and recurrent tamoxifen-resistant tumours (and a parallel study of a mouse xenograph model of tamoxifen resistance) an association of phospho-p38 and elevated ErbB2 with tamoxifen resistance was found [151].

JNK

JNKs 1, 2 and 3 are activated by MKK 4 and 7, which are substrates of MAPKKKs (for example, MEKK1/2 or TGFβ-activated kinase 1). JNK has a wide range of opposing functions within cells but is best known for its role in triggering apoptosis after exposure to cellular and environmental stresses [153]. Classically, JNK phosphorylates c-Jun, leading to the binding of c-Jun to c-Fos and the activation of activator protein-1 (AP-1), which can ultimately promote cell proliferation [154].

JNK-supported proliferation has been reported for a number of cultured breast cancer cell model systems; inhibition of JNK resulted in cell cycle arrest during G2/M in three different breast cancer cell lines, leading to DNA endoreduplication (cellular DNA content greater than 4N), decreased proliferation, and apoptosis [155]. In MCF-7 cells, JNK was found to promote cell growth after induction by IGF-1 and a presumed autocrine stimulation [156]. In both a clinical study of matched pairs of ER-positive tamoxifen-sensitive and tamoxifen-resistant human breast tumours [157] and a xenograph model of tamoxifen resistance [158], a positive association of activated/phosphorylated JNK with tamoxifen resistance has been observed. In contrast, JNK action has been reported to contribute in a number of independent ways to the three-dimensional organisation of normal mammary epithelial cells (that is, during acinus development). The actions of glucocorticoids in supporting the patency of mammary epithelial cell tight junction adhesions is well described [159]. Murtagh and colleagues [160] showed that glucocorticoids act to support such activities during mammary epithelial acinus formation by activating JNK signalling in a BRCA1-dependent manner. Inhibition of JNK action during acinus formation blocks the establishment of cell polarisation, the formation of tight junctions, the AP-1-dependent expression of tight junction component proteins and lumen clearance, allowing the formation of tumour-like disordered assemblies of cells; the latter cascade of events is ERK1/2-dependent and reversible with a MEK1 inhibitor (E McArdle, unpublished observation). Interestingly, it has been subsequently shown that c-Myc induces cell death in MCF-10A cells during acinus formation by inducing formation of a Scribble-bPIX-GIT1 complex that activates a Rac-JNK-c-Jun-Bim apoptotic pathway. Inhibition of ERK1/2 in this context

does not inhibit c-Myc-induced apoptosis, suggesting that c-Myc specifically uses the JNK pathway to induce cell death in ordered mammary epithelial cells [161]. These studies emphasise the tumour-suppressor-like activities of the JNK signalling pathway, at least in these cell culture model systems.

ERK5

The fourth MAP kinase of interest in this context is ERK5 [2]. Relatively, it is of large molecular size [162], with its activation being carried out by the MAPKK MEK5 [163], which is itself activated by MEKK2 or 3 [164]. While distantly related to, and sharing its mechanism of molecular action with, ERK1/2, gene knockout studies in mice show ERK5 to have non-redundant functions in heart development [165], vasculogenesis and angiogenesis [166] and endothelial cell survival [166]. Cell culture based experiments have pointed to ERK5 functioning at a molecular level in mitosis [167] and cell survival [168]. ERK5 is activated by growth factors [169], integrin engagement [170] and cell stress [167], and its important molecular targets would seem to include the induction of transcription of components of the transcription factor AP-1 (cJun [171] and Fos [172]) and activation of transcription factors of the myocyte enhancer family group (for example, MEF2C, a well characterised target [173]), and cMyc [174]. Serum and glucocorticod kinase is an interesting target kinase of ERK5 [175]; it may mediate cell survival influences of ERK5 to cellular stress [176]. Interestingly, in 20% of a human breast tumour set analysed, ERK5 levels were elevated and activated ERK5 (phospho-ERK5) was detected in many of the tumours. High levels of ERK5 were found to be an independent predictor of disease-free survival in this cohort and was associated with poor disease outcome [177]. Complementing these findings are, firstly, studies on ERK5 knockdown (using short hairpin RNA) in a human breast cancer cell line, which showed a sensitisation to the effects of ErbB 2 inhibitors [177]; secondly, studies in human breast cancer cell lines that associate constitutively active ERK5 with induced ErbB2, 3 and 4 activation [178]; and, thirdly, that such cells harbouring a dominant defective ERK5 show reduced proliferation rates, which may be due to failure to activate ErbB2 [178].

Future perspectives

We still do not have a full understanding of the key contributions made by MAPK signalling to the different stages of mammary gland development. But the clues we have suggest interesting possibilities: during pubertal ductal morphogenesis, quantitative temporal differences in ERK1/2 activity may trigger the decision to branch [7]; ERK1/2 signalling may be the final mediator of the proliferation associated with alveogenesis, of which β1-integrin is a significant driver [179]; glucocorticoid-JNK-cJun signalling may be the primary driver of tight junction 'closure' required at the transition from late pregnancy to lactation [160]; and JNK may also play a role in ductal development in the terminal end bud at puberty as judged from its role in other developmental epithelial systems [180,181]. We await developments with interest. At the other end of the scale is the challenge of benefiting from the prognostic measurement of 'activated MAPK' (most immediately activated ERK1/2 and ERK5) levels in breast cancer. With anti-phospho-MAPK antibodies of good quality available, robust measurements of 'activated/phosphorylated-MAPK' can be made. However, this assesses potential rather than actual MAPK activity. Future analyses where phospho-MAPK levels are co-assessed with known molecular outputs of MAPK action will provide a true measure of activity and will be useful both from a prognostic point of view and in defining therapeutic targets. Of great importance also are therapeutic strategies that target the contribution of intracellular signalling networks that support disease progression in human breast cancer. It is suspected that signalling from over-expressed ErbB2 through ERK1/2 contributes to disease progression, but some studies suggest that signalling pathways other than the ERK1/2 pathway may be of equal or greater importance in tumours exhibiting ErbB2 over-expression [26]. EGFR-ERK1/2 signalling plays a role in disease progression in some forms of tamoxifen-resistant disease [84], and VEGF receptor-ERK1/2 signalling has also been linked to disease progression [26]. Currently, monotherapies that target the receptor or ERK1/2 activity are predicted to be of limited value. Instead, combination therapies that target growth factor receptors, multiple signalling pathways and signalling targets point the way forward [83,84]; an example of this would be an EGFR/ErbB2 inhibitor plus a multi-signalling pathway inhibitor (for example, the Ras farensyltransferase inhibitor, which blocks ERK1/2 activation and mTor (mammalian target of rapamycin) signals (or a Src inhibitor [182]) plus a 'full oestrogen antagonist' (for example, fulvestrant)). Rapid development of successful strategies in this area is keenly awaited.

Competing interests

The authors declare that they have no competing interests.

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