The role of tyrosyl phosphorylated PAK1 in the synergetic effect of estrogen and prolactin in breast cancer cells

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A Dissertation

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by

Peter Oladimeji

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biology

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Dr. Maria Diakonova, Committee Chair

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College of Graduate Studies

The University of Toledo

August 2015
An Abstract of

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Even though serine-threonine kinase PAK1 is activated by estrogen and plays an important role in breast cancer, the role of PAK1 in estrogen response is not fully understood. We show here that in MCF-7 breast cancer cells, estrogen activates PAK1 through both ERα and GPER1 plasma membrane receptors and that cytoplasmic Etk/Bmx phosphorylates and activates PAK1 in response to estrogen. Tyrosines 153, 201 and 285 of PAK1, which we have previously implicated in prolactin signaling as sites of JAK2 phosphorylation, play a role in Etk-dependent PAK1 activation since phospho-tyrosyl-deficient PAK1 Y3F mutant was not phosphorylated and not activated by Etk in response to estrogen. We describe a signaling complex composed of pTyr-PAK1, kinase Etk, Gβ1, Gγ2 and/or Gγ5 subunits of heterotrimeric G protein, and βPIX (PAK-associated guanine nucleotide exchange factor) which, upon formation, further activates PAK1 in a positive feed-back manner. Activity of protein kinase A (PKA), the main kinase which phosphorylates Ser305 of ERα in response to estrogen, is reciprocally regulated by pTyr-PAK1 in estrogen-treated cells. However, in response to prolactin, Ser305-ERα is phosphorylated by pTyr-PAK1. Our data suggest that in cells exposed to
both prolactin and estrogen, Ser305-ERα is phosphorylated by both PKA and pTyr-PAK1 resulting in maximal signal. Furthermore, activation of S305-ERα leads to enhanced phosphorylation of Ser118-ERα, and eventually promotes cell proliferation. Thus, we introduce PAK1 as a common node for estrogen- and prolactin-dependent pathways which makes it an attractive target for anti-cancer therapy.

Differentiation-inducing factors 1-3 (DIFs 1-3), chlorinated alkylphenones identified in the cellular slime mold Dictyostelium discoideum, are considered anti-tumor agents because they inhibit proliferation of a variety of mammalian tumor cells in vitro. Although the anti-proliferative effects of DIF-1 and DIF-3 are well-documented, the precise molecular mechanisms underlying the actions of DIFs have not been fully elucidated. We examined the effects of DIFs and their derivatives on PAK1, a key serine-threonine kinase, which is activated by multiple ligands and regulates cell proliferation. It was found that some derivatives strongly inhibited PAK1 kinase activity in human breast cancer MCF-7 cells stably overexpressing PAK1. Among the derivatives, DIF-3(+1) was most potent, which directly inhibited kinase activity of recombinant purified PAK1 in an in vitro kinase assay. Furthermore, DIF-3(+1) strongly inhibited both cyclin D1 promoter activity and proliferation of MCF-7 and T47D breast cancer cells stably overexpressing PAK1 in response to prolactin, estrogen, epidermal growth factor and heregulin.

PAK1 which has been implicated in breast cancer was shown previously to be tyrosyl phosphorylated by prolactin (PRL)-activated Janus tyrosine kinase (JAK2). Although a role for both PRL and PAK1 in breast cancer is widely acknowledged, the mechanism is yet to be fully understood. In the present study, PRL-activated PAK1 stimulates the invasion of TMX2–28 human breast cancer cells through Matrigel. Three-
dimensional (3D) Collagen IV stimulates the secretion of the matrix proteases, metalloproteinase (MMP)-1 and -3 that is further enhanced by the PRL-dependent tyrosyl phosphorylation of PAK1. 3D Collagen IV also stimulates the expression and secretion of MMP-2, but in contrast to MMP-1 and -3, PRL/PAK1 signaling down-regulates MMP-2 expression and secretion. In contrast, MMP-9 expression and secretion are stimulated by 3D Collagen I, not Collagen IV, and are not affected by PRL but are down-regulated by PAK1. MMP-1 and -3 are required and MMP-2 contributes to PRL-dependent invasion. ERK1/2 signaling appears to be required for the enhanced expression and secretion of MMP-1 and -3 and enhanced PRL-dependent invasion. p38 MAPK and c-Jun N-terminal kinase 1/2 pathways participate in production of MMP-1 and -3 as well as in PRL/PAK1-dependent cell invasion. Together, these data illustrate the complex interaction between the substratum and PRL/PAK1 signaling in human breast cancer cells and suggest a pivotal role for PRL-dependent PAK1 tyrosyl phosphorylation in MMP secretion.
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# Table of Contents

Abstract iii  
Acknowledgements vi  
Table of Contents vii  
List of Figures viii  
List of Abbreviations xi  
I. INTRODUCTION 1  
II. MATERIALS AND METHODS 28  
III. RESULTS 39  
   A. The role of tyrosyl phosphorylated PAK1 in the synergetic effect of estrogen and prolactin in breast cancer cells 39  
   B. Derivative of differentiation-inducing factor-3 inhibits PAK1 activity and breast cancer cell proliferation 70  
   C. PAK1 regulates breast cancer cell invasion through secretion of matrix metalloproteinases in response to prolactin and three-dimensional Collagen IV 79  
IV. CONCLUSIONS AND PERSPECTIVES 103  
V. REFERENCES 107
List of Figures

Figure 1. Classical and alternative estrogen signaling pathways ..........................3
Figure 2. Schematic of important phosphorylation sites on ERα ..........................4
Figure 3. Overview of the main PRL signaling pathways .................................8
Figure 4. PRL and E2 cooperate to regulate gene expression and cell proliferation 11
Figure 5. PAK1 domain structure and phosphorylation sites .............................13
Figure 6. Overview of PAK1 activity in cells ..................................................14
Figure 7. PRL-dependent tyrosyl phosphorylation of PAK1 regulate PAK actions 19
Figure 8. GIT1/βPIX/PAK1/paxillin complex ..................................................23
Figure 9. Overview of PKA activation ............................................................25
Figure 10. Chemical structure of DIF derivatives ............................................32
Figure 11. Estrogen-activated PAK1 regulates breast cancer cell proliferation ....40
Figure 12. Estrogen activates PAK1 WT but not PAK1 Y3F ..............................41
Figure 13. PAK1 Y3F is functionally active ....................................................42
Figure 14. JAK2, c-Abl, ErbB or Src do not mediate estrogen-dependent PAK1 tyrosyl phosphorylation ........................................43
Figure 15. Etk phosphorylates and activates PAK1 in response to estrogen ....45
Figure 16. Both GPER1 and mERα are involved in PAK1 activation in response to estrogen .................................................................46
Figure 17. Both GPER1 and mERα are involved in PAK1 activation in response to estrogen .................................................................47
Figure 18. Gβγ subunits of G protein activate PAK1 in response to estrogen ....48
Figure 19. E2-activated PAK1 makes a complex with Gβγ subunits of G protein, Etk and βPIX.

Figure 20. βPIX facilitates E2-dependent PAK1 activation.

Figure 21. βPIX regulate estrogen-dependent PAK1 activation.

Figure 22. Selective inhibitors inhibit multiple MAPKs, PI3K, PKC, NFκB.

Figure 23. Reciprocal regulation of PAK1 and PKA in E2 signaling.

Figure 24. Ser305-ERα is phosphorylated by PAK1 in response to PRL.

Figure 25. Ser305-ERα is phosphorylated by PKA in response to E2 and by PAK1 in response to PRL.

Figure 26. Proposed mechanisms for the role of PAK1 in synergetic effects of PRL and E2 on ERα activation.

Figure 27. DIF-3 and its derivatives inhibit PAK1 kinase activity in cells.

Figure 28. DIF-3(+1) inactivates purified PAK1 in vitro.

Figure 29. DIF-3(+1) decreases cyclin D1 promoter activity.

Figure 30. DIF-3(+1) inhibits breast cancer cell proliferation.

Figure 31. Activation of PAK1 WT is stronger than PAK1 Y3F in response to PRL.

Figure 32. Maximal invasion of TMX2–28 cells in response to PRL requires tyrosyl phosphorylation of PAK1.

Figure 33. Collagens and PRL-dependent tyrosyl phosphorylation of PAK1 regulate secretion of invasive-relevant MMPs.

Figure 34. PAK1 is essential for secretion of invasive-relevant MMPs.

Figure 35. PRL regulate multiple MAPKs.
Figure 36. Selective Inhibitors Inhibit PRL-Activated Multiple MAPKs

Figure 37. UO 126 inhibitor does not inhibit PRL-activated Erk5

Figure 38. Maximal Invasion of TMX2–28 Cells in Response to PRL Requires MMPs 1, 2, 3, and 9 and MAPK Activities

Figure 39. PRL-Dependent Tyrosyl Phosphorylated PAK1 and 3D Collagen IV Regulate MMP-1 and MMP-3 Production and Invasion via MAPK Pathways Schematic representation of the proposed working model
List of Abbreviations

ATP……………….. Adenosine triphosphate
BSA……………….. Bovine serum albumin
CAK……………….. CDK-activating kinase
CDK……………….. Cyclin-dependent kinase
cDNA……………… Complementary deoxyribonucleic acid
CRIB……………….. Cdc42/Rac interactive binding
E2……………….. 17β-estradiol
EGF……………….. Epidermal growth factor
ER……………….. Estrogen receptor
ETK……………….. Endothelial/epithelial tyrosine kinase
FBS……………….. Fetal bovine serum
FGF……………….. Fibroblast growth factor
GPER1……………… G protein coupled estrogen receptor 1
HRG……………….. Heregulin
IB……………….. Immunoblotting
IGF-1……………… Insulin-like growth factor-1
IP……………….. Immunoprecipitation
JAK2……………….. Janus kinase 2
JNK……………….. c-Jun N-terminal kinase
MAPK……………… Mitogen-activated protein kinase
PAK1……………….. p21-activated kinase 1
PBD..............p21-binding domain
PBS..................Phosphate buffered saline
PDGF..............Platelet derived growth factor
PEI..................Polyethyleneimine
PH..................Pleckstrin homology
PI3K..............Phosphatidylinositol 3 kinase
PIX..............PAK-interacting exchange factor
PMSF..............Phenylmethanesulphonylfluoride
PRL..............Prolactin
PRLR..............Prolactin receptor
pY..................Phosphotyrosine
SDS-PAGE..............Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SH2..............Src homology 2
SH3..............Src homology 3
STAT..............Signal transducer and activator of transcription
VEGF..............Vascular endothelial growth factor
WT..............Wild type
I. INTRODUCTION

Estrogen

Estrogen is a steroid hormone and is a key regulator of growth, differentiation and normal development of the reproductive and central nervous system. Estrogen functions in a variety of target tissues such as vascular tissues, skeletal system and mammary gland (Gustafsson, 2003). There are three naturally occurring estrogen—estrone (E1), 17β-estradiol (E2) and estriol (E3)—all of which are derived from cholesterol (Kuiper et al., 1997). Of the three, E2 is the most dominant and most potent in humans (Kuiper et al., 1997). Actions of E2 are mediated predominantly by binding to estrogen receptor (ER). ER is a member of the superfamily of nuclear receptors and is a ligand-activated transcription factor. In an inactive state, ER is complexed with heat-shock proteins and immunophilins. This inhibition is relieved upon E2 binding leading to conformational change and activation of ER (Pratt and Toft, 1997). Active ER isoforms homodimerize or heterodimerize and bind with high affinity to specific DNA sequence known as estrogen response elements (ERE) located in the regulatory region of estrogen target genes (Loven et al., 2001; Pratt and Toft, 1997). The E2-ER complex also binds nuclear receptor coactivators and repressors that modulate ER transcriptional activity. Some of the cofactors recruited by the E2-ER complex include SRC1, GRIP1, AIB1, CBP/p300, TRAP220, PGC1, p68 RNA helicase, and SRA (Rosenfeld and Glass, 2001; Shao and Brown, 2004). Apart from the classical ER-ERE gene regulation, ER can regulate non-ERE containing genes by regulating other classes of transcription factor through protein-protein interactions. In fact about one-third of ER regulated genes do not contain ERE. A
typical example of such is the interaction of ER with activator protein 1 (AP-1) transcription factor for the regulation of ovalbumin, IGF-1, Collagenase and cyclin D1 genes. Other transcription factors regulated by ER include Sp1 and NF-kB to regulate E2F1 and interleukin 6 genes respectively (Liu et al., 2002; Sabbah et al., 1999; Umayahara et al., 1994; Webb et al., 1995).

ER has two subtypes; ERα and ERβ encoded for by different genes and have strong similarities in the DNA- and ligand-binding domain (Green et al., 1986; Kuiper et al., 1996; Mosselman et al., 1996). The two receptors elicit different functions as evidenced in knockout mouse models of each ER subtype (Couse and Korach, 1999; Couse et al., 1997). The ligand-dependent transcriptional activity of ERα is stringently regulated by the constitutive activation function 1 (AF-1) in the N-terminus and the hormone-dependent activation function 2 (AF-2) located in the ligand binding domain (Berry et al., 1990). AF-2 domain has been reported in ERβ as well based on sequence homology and in vitro studies; however, the AF-2 in ERβ has a role different from that in ERα (Kraus et al., 1995; Nilsson et al., 2001). Besides the differences in functions, ERα and ERβ are distributed in tissues differently. ERα is the predominant subtype expressed in breast, endometrium, uterus, cervix and vagina, while ERβ is detected in non-classic target tissues like kidney, bone marrow, brain, prostate, ovary, spleen, testis, lung, hypothalamus and thymus (Couse et al., 1997), hence our study will focus on signals mediated by ERα in breast cancer cells.

It is now clear that, through phosphorylation, alternative signaling pathways can regulate ER functions. Extracellular signals such as EGF, IGF-1, TGF-β, insulin, heregulin, dopamine, interleukin-2 can mimic the effects of E2 to regulate ER target
genes (Ignar-Trowbridge et al., 1996; Newton et al., 1994; Patrone et al., 1998; Pietras et al., 1995; Power et al., 1991) (Figure 1). In most instances, the regulation of ER by these extracellular signals is through activation of different kinases including PKA, PKC,
MAPKs, CDK2, CDK7, c-Src, IKKα, Akt, pp90rsk1, GSK3 and PAK1 that can directly phosphorylate ER and its cofactors (Maggi, 2011). All of the steps involved in the transcriptional regulation of ER targets genes, i.e. ligand binding, ER dimerization, DNA binding and cofactor recruitment are controlled by the phosphorylation state of ER. E2 treatment is a potent stimulator of S118 phosphorylation. The kinase responsible for S118 phosphorylation is not clear; however, ERK1/2, CDK7, IKKα and GSK3β are possible candidates (Chen et al., 2002; Medunjanin et al., 2005; Park et al., 2005) (Figure 2).

Figure 2. Schematic of important phosphorylation sites on ERα. Multiple phosphorylated sites in ERα have been identified by a variety of approaches as depicted in the figure. The schematic shows different structural (A, B, C, D, E) and functional domains: activation function 1 (AF1), DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD) of human ERα. Modified from Murphy et al. 2011

Phosphorylation of amino acids 236 in the DNA binding domain by PKA, 294 in the hinge region by proline-directed kinase and 305 in the ligand binding domain by PKA and PAK1 are important phosphorylation site on ERα for its transcriptional activity. In addition, within the A/B domain of the ERα, phosphorylation of serines 104 and 106 by
CDK2, 154 by AKT and 167 by multiple kinases like RSK1, K1, AKT, IKKε and CK2 are of similar importance (Le Goff et al., 1994; Murphy et al., 2011) (Figure 2). S118 is a widely studied target, and phosphorylation on this residue facilitates ERα interaction with coactivators such as CREB binding protein to mediate ligand-dependent and ligand-independent activation of the receptor. The significance of ERα S305 phosphorylation is becoming more apparent through in vitro and clinical studies. S305 phosphorylation by both PAK1 and PKA results in enhanced transactivation of ERα. More importantly, in clinical studies there is a correlation between breast cancer recurrence and phosphorylation of ERα S305. More interestingly, E2-independent ERα S305 phosphorylation is suggested to contribute to hormone therapy resistance (Bostner et al., 2010; Holm et al., 2009a; Murphy et al., 2011; Wang et al., 2002). As previously described, E2 binds ER to initiate a sequence of events that ultimately lead to the regulation of target genes.

E2 exert some of its effects on target genes through the binding of ER to ERE leading to gene transcription and translation. Several effects of E2 are rapid that they cannot depend on RNA activation and protein synthesis. Such actions include rapid activation of kinases and phosphatases, and ion fluxes modulation across plasma membrane (Deecher et al., 2003; Razandi et al., 2004). The observed rapid actions of E2 in the bone, breast and nervous system suggest that E2 elicit non-classical effects through plasma membranes-localized ERα that are linked to intracellular signal transducers (Figure 1). The rapid actions of E2 are not inhibited by transcription inhibitors suggesting that these effects are independent of ERα transcriptional activity. This is supported by the evidence that membrane impermeable E2 can elicit quick actions, within seconds to
minutes, similarly to membrane permeable E2 on signal transduction pathways (Dominguez and Micevych, 2010; Vasudevan and Pfaff, 2007). E2 actions emanating from the plasma membrane are referred to as alternative signaling pathway. About 5-10% of ERα is translocated to the plasma membrane in target cells. ERβ is also present at the plasma membrane in cardiovascular cells. In breast cancer cells, it is mainly ERα that is found at the plasma membrane (Acconcia et al., 2005). ERα and ERβ are localized to calveolae raft in the plasma membrane alongside several other signaling molecules such as G proteins, growth factor receptors, and other non-receptor tyrosine kinases that facilitates rapid signaling. The E domain of the ER interacts with calveolin-1 that is essential for the transport of ER to calveolae (Acconcia et al., 2005; Li et al., 2003a).

Because ER does not contain transmembrane domain, they undergo palmitoylation for them to be anchored to the plasma membrane. ER undergoes palmitoylation within the E domain containing the palmitoylation motif (Acconcia et al., 2005; Pedram et al., 2007). Similar palmitoylation motifs are found in the E domain of progesterone and androgen receptor (Pedram et al., 2007).

Several models have been described for the rapid actions of E2 through membrane localized ER, but not all E2 action can be attributed to ERα and ERβ. In certain cases, ER antagonist could not inhibit certain rapid signaling events. This led to the hypothesis that other membrane bound estrogen-responsive receptors exist. Recently, a G protein-coupled receptor, GPR30, was found to mediate rapid E2 effects in SKBr3 breast cancer cell line (Filardo et al., 2002) (Figure 1). Additionally, rapid activation of Erk1/2 by E2 was lost in MDA-MB-231 cells which lack GPR30; however, such action was recovered by expression of GPR30 (Filardo et al., 2002). These findings suggested
that GPR30 is a receptor for E2 and led to the renaming of the receptor currently referred to as G protein coupled estrogen receptor 1 (GPER). GPER has been seen distributed in normal and malignant human tissues with high level of expression found in the heart, lung, liver, ovary and brain. Several primary breast cancer and lymphomas also express GPER (O'Dowd et al., 1998). GPER functions have been implicated in the reproductive system, nervous system, immune system, cardiovascular system, renal system, pancreatic function, bone growth, cancer growth—including breast cancer—and metastasis (Carmeci et al., 1997; Prossnitz and Barton, 2011). Increased expression of GPER in breast cancer suggests a role for the receptor in cancer progression, especially in cases of ER negative breast cancer. GPER1 may compensate for the loss or inhibition of ER by antagonists. The identification of GPER1 has added another layer of complexity to the understanding of E2 action. The downstream signaling cascade for GPER is just being elucidated and at present poorly understood. It is important to delineate E2 action mediated by ER\(\alpha\) and GPER, identify potential cross talks with other ligands such as prolactin, and contribution of GPER to breast cancer progression.

**Prolactin**

Prolactin (PRL) is a 23kDa polypeptide hormone primarily synthesized and secreted from the anterior pituitary. The hormone was named based on its role in initiating and sustaining milk production from the mammary glands. However, about 300 other biological functions of PRL have been discovered including promoting mammary gland development, cell proliferation, differentiation and survival (Bernichtein et al., 2010). Apart from the anterior pituitary, PRL is also secreted in multiple tissues including mammary epithelium, adipocytes, the prostate and immune cells where it is regulated in a
cell specific manner and functions in an autocrine/paracrine fashion (Ben-Jonathan et al., 2008). Actions of PRL are mediated by PRL receptor (PRLR). PRLR is a single-pass transmembrane protein that belongs to the cytokine receptor superfamily, and is most markedly expressed in mammary epithelium (Bazan, 1989). Seven isoforms of the human PRLR have been identified. These isoforms result from the alternative splicing of the primary transcript as well as transcription starting at alternative initiation sites (Clevenger et al., 2009). The major differences between the PRLR isoforms are in the length and composition of the cytoplasmic region, the extracellular domain are very similar. The most common of the isoforms is the long PRLR (Clevenger et al., 2009). PRLR has no endogenous kinase activity, thus require nonreceptor tyrosine kinases to initiate downstream actions of PRL. One of such kinases and the best characterized downstream of PRL signaling is JAK2 (Campbell et al., 1994; Rui et al., 1994a) (Figure 3). JAK2 is

**Figure 3. Overview of PRL signaling pathways.** The major signal transduction pathways of PRL are the JAK2/STAT1,3,5, JAK2/PAK1, PI3K/AKT and MAPK which are activated by PRL in a cell-selective manner to regulate cell physiology.
activated and autophosphorylated on tyrosines 1007 and 1008 upon PRL binding to PRLR, thus resulting in PRLR phosphorylation (Feng et al., 1997; Rui et al., 1994b).

PRL activates several other signaling pathways which include MAPK, PI3K, AKT, Src family kinases, protein kinase C, Rho GTPases, Rush (Das and Vonderhaar, 1996a; Das and Vonderhaar, 1996b; Waters and Rillema, 1989).

PRL secretion is up-regulated in human breast cancers, adding to the evidence of the involvement of PRL in breast cancer progression (McHale et al., 2008). 70-90% breast tumors express PRLR; in addition, elevated levels of PRL and PRLR have been observed in human breast tumor compared to normal breast tissue (Touraine et al., 1998). JAK2-STAT5 pathway plays an immense role in breast cancer. Increased serum PRL level modestly correlates with higher cancer risk in pre- and post-menopausal women and poor prognosis, and breast cancer metastases (Mujagic and Mujagic, 2004; Tworoger and Hankinson, 2008). Epidemiologic evidence corroborate this by showing that women with elevated blood PRL level have worse treatment and survival outcomes, and such patients are less responsive to chemotherapeutic drugs like taxol, cisplatin, vinblastine and doxorubicin (Tworoger and Hankinson, 2008). In fact, PRL acts as a survival factor in breast cancer by activating PI3K-AKT survival pathway and upregulating Bcl-2 in breast cancer cells. Furthermore, PRL promotes neoplastic transformation by increasing cell proliferation and potentiates transition to invasive carcinoma. PRL promotes metastatic properties of breast cancer cells by regulating cytoskeleton rearrangement, hence, enhancing membrane ruffling and cell motility and invasion (Mujagic and Mujagic, 2004). PRL has been described as a chemoattractant for breast cancer cell, but little is known of the mechanism underlying the process. Two mechanisms of PRL-regulated cell


motility were recently proposed. Firstly is the regulation of PAK1-Filamin A axis, and secondly is the regulation of cell adhesion turnover (Hammer et al., 2015b; Hammer et al., 2013). PRL could suppress metastatic tumor progression in contrast to its enhancing action in early tumor stages (Sato et al., 2014; Tran et al., 2010). Such contrasting characters are also exemplified by TGF-β and E2 (Becker et al., 2004; Dong et al., 2007; Lee et al., 2010).

Breast tissues secret PRL which functions in an autocrine / paracrine fashion, and for this reason the previous attempt to inhibit pituitary PRL as a method of treating breast cancer was futile. Moreover, the mechanism by which PRL promotes breast cancer and confers resistance to anticancer drugs is not completely understood. It is, therefore, important to investigate the mechanism through which PRL functions to promote breast cancer progression, and further delineate the mechanism of crosstalk with estrogen (the main hormone involved in breast cancer progression) in order to properly treat the disease.

**Prolactin and estrogen crosstalk**

PRL and E2 have long been implicated in the pathogenesis of breast cancer. Even though the two hormones are different in structure, receptor characteristics and signaling mechanism, there is a crosstalk between them (Figure 4). On one hand, PRL signaling is known to increase the transcription of ERα and ERβ, and phosphorylation of ERα (Arendt and Schuler, 2008; Frasor and Gibori, 2003; Glaros et al., 2006), while on the other hand E2 induces the transcription of both PRL and PRLR in breast cancer cells (Dong et al., 2006; Duan et al., 2008; Swaminathan et al., 2008). In a study where MCF-7 cells were engineered to overexpress PRL, increased ERα expression and estrogen
responsiveness were observed as determined using markers including proliferation, ERE-luciferase activity, and increased expression of target genes such as cyclin D1, progesterone receptor, and Bcl-2 (Gutzman et al., 2004a). Estrogen can potentiate PRL-activated Stat5 activity in some mammary cells, including some breast cancer cell lines.

Figure 4. PRL and E2 cooperate to regulate gene expression and cell proliferation. Three mechanisms are suggested for cooperative gene regulation by PRL and E2: (A) PRL potentiates E2-induced gene transcription by inducing phosphorylation of ER leading to increased transcription of ER target genes, as observed for EGR3. (B) E2 enhances PRL-induced gene transcription by further activating the ERK1/2 pathway, which causes increased transcriptional activity of PRL-stimulated transcription factors, as found for EGR1. (C) PRL and E2 cooperate to regulate the same target genes via either direct or indirect interactions at the promoter regions. Additive regulation was observed for several genes, including IER3, AREG, CITED2 and TP53INP1. From Rasmussen et al. 2010a.
These indicate that PRL is an important modulator of E2 actions in breast cancer and such interactions can result in augmentation or synergism between the two hormones.

**p21-Activated kinase 1**

p21-Activated kinase (PAK) is a family of serine/threonine kinases that consists of six members divided into group I (PAK1-3) and group II (PAK4-6) based on sequence similarities and functional features such as an autoinhibitory domain present in group I but not group II (Bokoch, 2003). Group I PAKs share other characteristics like p21-binding domain (PBD), proline rich regions and kinase domain. PAKs have similar structural organization, including an N-terminal regulatory domain and a catalytic domain in the C-terminal. The conserved N-terminus of PAK1 has a GTP-binding domain (GBD) which binds the GTPases Rac and cell division cycle 42 (Cdc42). Group I PAKs, in their regulatory domain, have autoinhibitory domain (AID) that partially overlaps with the GBD (Bokoch, 2003; Lei et al., 2000) (Figure 5). Among group I PAKs, there are conserved proline rich regions in the regulatory domain that interact with SH3-containing adapter protein such as Nck and Grb2 as well as guanine exchange nucleotide factor PIX/COOL. Additionally, there are three nuclear localization sequences (NLS) and multiple phosphorylation sites in the regulatory domain. The kinase activity of group I PAKs resides in C-terminal end with highly conserved amino acids. Group II PAKs lack the autoinhibitory domain and proline rich regions (Lei et al., 2000; Parrini et al., 2002). PAK1 is the most-well characterized family member and is expressed in a variety of normal tissues. Recently, PAK1 was shown to be a phosphorylated on Tyr153,
PAK1 plays a significant role in regulating cell motility by mediating extracellular stimuli and actin and cell adhesion dynamics. Additionally, PAK1 functions in cell cycle progression, gene transcription, cell proliferation, survival and apoptosis (Bokoch, 2003; Kumar et al., 2006; Zhao and Manser, 2005) (Figure 6). Despite its diverse roles in cells, the molecular mechanism underlying PAK1 activities remains poorly understood.

The various cellular processes regulated by PAK1 rely on the finely tuned activation of specific pathways in response to various extracellular stimuli, subcellular localization and protein-protein interactions. Cellular fractionation and microscopy has shown PAK1 localization primarily in the cytoplasm. However, upon stimulation by
growth factors, PAK1 associates with plasma membrane through interaction with Nck and Grb2. Upon stimulation, SH2 domain on NCK and Grb2 interact with phosphorylated tyrosines on receptor tyrosine kinases, while their SH3 domain interact with proline motif on PAK1 (Bokoch et al., 1996; Daniels et al., 1998; Lu et al., 1997). Inactive PAK1 is restricted to the cytoplasm as a homodimer where the AID of one PAK1 molecule obstructs the kinase domain of an adjacent PAK1 and vice versa to remain in an inactive conformation. PAK1 localization to the membrane induces a conformational change which leads partial opening of the dimeric complex, hence, partial
activation of PAK1 (Pirruccello et al., 2006). Partially open PAK1 are more responsive to stimulation by Rac1 and Cdc42 (Parrini et al., 2009; Pirruccello et al., 2006). Binding of small Rho GTPases (Rac1 and Cdc42) to the GBD on PAK1 is required for the relief of autoinhibition and full activation PAK1, keeping PAK1 in an active conformation. Full activity of PAK1 can also be achieved through interaction with PIX at cell adhesions (Manser et al., 1998). PAK1-Rho GTPase binding induces PAK1 autophosphorylation/transphosphorylation on serine 144 and threonine 423, the major PAK1 autophosphorylation site regulating PAK1 kinase activity in the kinase domain (Parrini et al., 2009) (Figure 5). PAK1 activity can also be regulated in a GTPase-independent manner. Direct interaction of PAK1 with lipids such as sphingosine and lysophosphatidic acid can induce PAK1 activity (Bokoch et al., 1998). Phosphorylation of PAK1 on Thr423 by PDK1 may play an important role in the recruitment of PAK1 to the plasma membrane by Nck (King et al., 2000). Only a few negative regulators of PAK1 have been identified which includes PAK-interacting protein, neuron-specific kinase Cdk5, and the tumor suppressor merlin. These negative regulators inhibit PAK1 by binding and keeping PAK1 in an inactive conformation, prevent Cdc42 and Rac1 binding to PAK1, or actively dephosphorylating key activation sites on PAK1 (Banerjee et al., 2002; Hirokawa et al., 2004; Kissil et al., 2003; Thiel et al., 2002; Xiao et al., 2005).

PAK1 translocates into the nucleus depending on the cell cycle phase. Endogenous PAK1 localizes inside the nucleus of 100% of cells during metaphase and about 20% during interphase (Li et al., 2002). Nuclear translocation of PAK1 is also promoted in response to stimulus, for instance PRL (Tao et al., 2011). PAK1 nuclear
import is facilitated by interaction with the Dynein light chain LC8. The nuclear localization of PAK1 has been shown to be essential in zebrafish development (Lightcap et al., 2009). There is evidence of the involvement of PAK1 in the regulation of genes expression through interaction with promoters and/or transcription factors. For instance PAK1 associates with the enhancer regions of phosphofructokinase-muscle isoform and nuclear factor of activated T-cell (NFAT) to directly regulate gene expression. Activated PAK1 localizes to centrosome in mitotic cells and phosphorylate histone H3 specifically (Li et al., 2002). Localization of PAK1 to the centrosome suggests PAK1’s involvement in kinetochore to spindle attachments; dysregulation of PAK1 may account for aneuploidy and other chromosomal abnormalities observed in tumor cells. Increase in active PAK1 nuclear localization has been linked to the development of tamoxifen (TAM) resistance in breast cancer (Holm et al., 2006; Rayala et al., 2006a). Other significances of PAK1 nuclear localization are still under investigation.

PAK1 deregulation results in ERα transactivation and inhibition of TAM sensitivity in ERα positive breast cancer cells (Rayala et al., 2006a). Active PAK1 phosphorylates Ser305 on ERα leading to constitutively phosphorylation of ERα S118 independent of E2. The cooperative phosphorylation of S305 and S118 promotes ERα transcriptional activity (Rayala et al., 2006a). Growth factor signaling can activate PAK1 and confer TAM resistant phenotypes. AND-34/BCAR3, upstream activators of PAK1, also induce TAM resistance in a Rac1-dependent manner (Cai et al., 2003). Inhibition of PAK1 by inhibitor FK288 results in shrinkage of tumor in TAM resistant cells (Hirokawa et al., 2005). These suggest that PAK1 plays a pivotal role in TAM resistance in breast cancer. Apart from PAK1, protein kinase A (PKA) is also known to phosphorylate ERα
Ser305 (Schreihofe et al., 2001; Tsai et al., 2004). Phosphorylation of ERα Ser305 either by PAK1 or PKA keeps ERα in an active conformation, so that TAM sensitivity is diminished.

Cancer progression is a multistage process. A cancerous cell has to overcome multiple obstacles in order to survive and thrive. Cell microenvironment is tightly regulated to keep cells healthy and rid of unhealthy ones. Despite this, cancerous cells must survive in their initial environment, detach from neighboring cells, invade into extracellular matrix, colonize and establish in new areas. Remarkably, PAK1 has been described in each of these stages of cancer progression. Aberrant PAK1 signaling has been linked to malignancies including bladder, colorectal, gastric, lymph, brain, ovarian and breast cancers (Dummler et al., 2009). The PAK1 gene is localized within the 11q13 region, and 11q13.5-q14 amplifications are found in bladder, ovary and breast cancer (Ye and Field, 2012). Other abnormalities frequently reported include elevated levels of mRNA and protein and enhanced activity of PAK1 (Balasenthil and Vadlamudi, 2003). Interestingly, experimental manipulations and clinical studies have paralleled the overexpression of active PAK1 and its accumulation in the nuclei of malignant cells with aggressive cancer phenotype and poor breast cancer prognosis in patients (Holm et al., 2006; Rayala et al., 2006a; Vadlamudi et al., 2000; Wang et al., 2006). Furthermore PAK1 expression, and more importantly, phosphorylated PAK1 was significantly associated with ovarian tumor grade, disease-free and overall survival of patients (Siu et al., 2010). Because PAK1 is a major component of the ongogenic pathway in breast cancer, understanding the mechanism of actions has been central to breast cancer studies.
Interference of PAK1 or its downstream targets may be a plausible therapeutic approach in the treatment of breast cancer.

**PAK1 tyrosyl phosphorylation**

Tyrosyl phosphorylation of PAK1 regulates an array of PAK1 activity in cells. In response to PRL, it has been demonstrated that PAK1 is a novel substrate of the JAK2 tyrosine kinase and that PRL-activated JAK2 phosphorylates PAK1 in vivo. Through mass spectrometry and 2D peptide mapping, PAK1 tyrosines 153, 201, and 285 were identified as sites of JAK2 tyrosyl phosphorylation. JAK2-dependent PAK1 tyrosyl phosphorylation plays an important role in cell survival and in the regulation of cyclin D1. We demonstrated that in the presence of PRL, the kinase activity of PAK1 WT was significantly greater than PAK1 Y3F (PAK1 mutant in which the three JAK2 phosphorylation sites, tyrosines 153, 201 and 285 were mutated to phenylalanine) in MCF-7, T47D and TMX2–28 breast cancer cell lines. Heregulin (HRG), a ligand for HER3 (human epidermal growth factor receptor-3) and HER4 (human epidermal growth factor receptor-4), activates both PAK1 WT and PAK1 Y3F to the similar extent confirming that PAK1 Y3F is catalytically active (Hammer et al., 2013; Rider et al., 2013b; Rider et al., 2007; Tao et al., 2011). Tyrosyl phosphorylation of PAK1 (pTyr-PAK1) enhances such important PAK1 functions as kinase activity and the ability to form protein/protein interactions. Both of these PAK1 activities are important for adhesion, motility, and invasion of breast cancer cells in response to PRL (Hammer et al., 2015b; Hammer et al., 2013; Rider et al., 2013b). pTyr-PAK1 also regulates cyclin D1 promoter activity in response to PRL (Tao et al., 2011). Similarly to PRL treatment, irradiation of lung cancer cells also leads to phosphorylation of tyrosines 153, 201 and
285 by JAK2 resulting in increases in PAK1 stability, PAK1/Snail binding, EMT and radioresistance of lung cancer cells (Kim et al., 2014). In addition to its kinase activity, PAK1 acts as a scaffold for many proteins including Raf-1, MEK and Erk (Sundberg-Smith et al., 2005) and this PAK1 activity depends on its ability to initiate protein–

**Figure 7. PRL-dependent tyrosyl phosphorylation of PAK1 regulate PAK actions.** PRL stimulates kinase activity of PAK1 and PAK1 ability to form protein–protein interaction. PRL binding induces dimerization of the PRLR and subsequent activation of JAK2. Active JAK2 phosphorylates PAK1 on three tyrosines, Tyr 153, 201 and 285. Tyrosyl phosphorylation of PAK1 enhances both a Rac-dependent and Rac-independent PAK1 kinase activity, and also the ability for PAK1 to act as a molecular scaffold. From Hammer and Diakonova, 2015

...protein interactions (Figure 7). Presumably, tyrosyl phosphorylated PAK1 may create additional docking sites to recruit SH2-domain containing proteins in order to enhance activation of recruited proteins and amplify PRL-dependent signaling (Figure 7). In such
case, pTyr-PAK1 will be able to recruit additional proteins to amplify downstream signals. Tyrosyl phosphorylated PAK1 have been implicated in phagokinesis, a cellular process that combines cell motility and phagocytosis. Cell ruffling, a process dependent on actin dynamics and required for directional cell migration, is also regulated by pTyr-PAK1 (Rider and Diakonova, 2011; Rider et al., 2007). Other important processes regulated by pTyr-PAK1 include adhesion turnover—the initial steps in cell motility—and cell invasion—crucial step in cancer metastasis (Hammer et al., 2015b; Rider et al., 2013b). These findings underscore the significance of tyrosyl phosphorylation of PAK1 in fine-tuning PAK1-regulated cellular processes, and implication in cancer progression.

In addition to JAK2, nonreceptor tyrosine kinase Etk/BMX can tyrosyl phosphorylate PAK1 and induce PAK1 kinase activation (Bagheri-Yarmand et al., 2001). Epithelial and endothelial tyrosine kinase (Etk, also called Bmx) belongs to the Tec family of non-receptor tyrosine kinases, which also includes Itk/Emt/Tsk, Tec, and Btk/Atk. The Tec family of kinases including Etk are characterized by an N-terminal Tec homology domain located downstream of a pleckstrin homology (PH) (Rawlings and Witte, 1995; Tamagnone et al., 1994). Furthermore, Etk contains Src homology-3 (SH3) and -2 (SH2) domains, and a kinase domain (Qiu et al., 1998b).

Etk participate in signal transduction in response to activation of growth factors or cytokines receptors, G-protein coupled receptors, antigen-receptors and integrins. They are also regulated by many non-receptor tyrosine kinases such as Src, JAK, Syk and FAK families (Qiu and Kung, 2000). Etk was demonstrated to activate Rho and serum response factor in response to Gα12/13 activation (Mao et al., 1998). Btk, a related Tec family member, binds directly to both Gα and Gβγ subunits (Hendriks et al., 2014).
Another member of this family, Tec, is activated by Gβγ subunits in response to thrombin (Hamazaki et al., 1998). Btk, the prototype for this family of kinases, is found to be important in a variety of signal transduction processes such as radiation-induced apoptosis and interleukin-induced growth (Kawakami et al., 1997; Siliciano et al., 1992; Uckun et al., 1996). Because previously described Btk family kinases are mainly expressed in hematopoietic cells, our understanding of the role of Btk family kinase signaling is confined to hematopoietic cells. Etk, however, is widely expressed in epithelial cells (Tamagnone et al., 1994).

Both PAK1 and Etk have been implicated downstream of PI3-kinase in human cancer cells, but the nature of the relationship between PAK1 and Etk remains elusive. Bagheri-Yarmand et al. demonstrated that PAK1 is a target of Etk, and that Etk activity is required for mammary epithelial cancer cell proliferation (Bagheri-Yarmand et al., 2001). Because PAK1, Etk and E2 have all been implicated in cell survival and proliferation, it is therefore important to understand the interplay of these three components in breast cancer cell proliferation.

Additionally, in constitutively activated v-Erb receptor-transformed cells, a very active and tyrosyl-phosphorylated form of PAK1 was found (McManus et al., 2000). Another PAK family member, PAK2, is regulated by tyrosyl phosphorylation. Src kinases phosphorylated PAK2 at Tyr 130 (Renkema et al., 2002). In contrast, another non-receptor tyrosine kinase, c-Abl, phosphorylates PAK2, but causes a decrease in PAK2 kinase activity. All these observations suggest that tyrosyl phosphorylation of PAK1 plays an important role in regulating PAK1 actions and elicits different activity of PAK1 depending on specific tyrosine kinase.
**p21-Interacting exchange factor β**

PIX (PAK1-interacting exchange factor) proteins were isolated as components of adhesion and direct binding partners of PAK1. Of the two forms that exist, αPIX is expressed primarily in hematopoetic cells and muscle, while βPIX is ubiquitously expressed (Bagrodia et al., 1998; Manser et al., 1998; Oh et al., 1997). βPIX is a member of the Dbl (diffuse B-cell lymphoma) family of Rho-GEFs and are characterized by the presence of tandem Dbl homology (DH) and Plekstrin homology (PH) domains. βPIX contain several protein–protein interaction motifs that dictate its localization and function. Importantly, both PIX contain N-terminal SH3 domain which binds with high affinity to proline-rich sequences in PAK1, c-Cbl and Rac1 (Frank and Hansen, 2008; Mayhew et al., 2007). βPIX and PAK1 form a signaling complex that mediates a number of cellular activities including cell adhesion, cell motility and mitotic checkpoint regulation (Mayhew et al., 2007) (Figure 8). It has been demonstrated that PIX is important for the sequestration of PAK1 to focal complexes and focal adhesions, and proposed that the two proteins in proximity to membrane-anchored Rac1/Cdc42 forms a PIX/PAK1/Rac1/Cdc42 complex that enhances PAK1 activation through the GEF activity of PIX (Bagrodia et al., 1999; Li et al., 2003b; Manser et al., 1998; Rosenberger and Kutsche, 2006).

In breast cancer cells, constitutively activated PAK1 was found to be mislocalized to large atypical focal adhesions. In that study, PAK1 localization to atypical focal adhesions requires its association with PIX; and experimental disruption of the PAK1-PIX interaction caused a displacement of PAK1 from focal adhesions as well as a
dramatic decrease of PIX and paxillin in these cellular adhesions and PAK1 activity (Stofega et al., 2004). PIX proteins associate with G protein coupled receptor kinase-interacting target 1 (GIT1), a GTPase activating protein (GAP) for Arf, that targets adhesion complexes by binding to paxillin (Turner et al., 1999). βPIX and GIT1 can dimerize as a part of a larger signalsome to regulate localization to sites of adhesion. Mutations that disrupt either GIT-βPIX association or βPIX homodimerization result in diffuse cytoplasmic localization of both proteins underlying the significance of βPIX-GIT1 interaction (Kim et al., 2001; Loo et al., 2004; Paris et al., 2003; Premont et al., 2004). In addition, PAK1/βPIX/GIT1 complex have been observed at the centrosome.
where PAK1 phosphorylate Aurora A and regulate mitotic checkpoint (Zhao et al., 2005). Hammer et al. demonstrated that tyrosyl phosphorylation of PAK1 increases the ability of PAK1 to bind βPIX and GIT1 (Hammer et al., 2015b). PAK1 is an important component of this complex and formation of the four-molecule PAK1/βPIX/GIT1/paxillin signaling module transiently targets PAK1 to the sites of adhesion where PAK1 can get activated (Brown et al., 2002; Zhao et al., 2000) (Figure 8).

βPIX, like PAK1, is upregulated in some breast cancer, and promotes growth in MCF-7 breast cancer cells by directly binding to the SH3 domain of PLCγ and enhancing phospholipase activity downstream of the PDGF receptor (Ahn et al., 2003; Bae et al., 2005). In response to chemoattractants, Gβγ dimer binds directly to PAK1, which simultaneously associates with αPIX (PAK-associated guanine nucleotide exchange factor) to form a Gβγ/PAK1/αPIX/Cdc42 complex leading to PAK1 activation (Li et al., 2003b).

**Protein kinase A**

Protein Kinase A (PKA: also known as cAMP-dependent protein kinase) is a multi-unit protein kinase and a member of the serine-threonine protein kinase superfamily. PKA mediates signal transduction of G-protein coupled receptors through its activation upon adenosine 3’5’-cyclic monophosphate (cAMP) binding. cAMP is generated from ATP by adenylate cyclases, (AC), and regulated by extracellular signals (Hanoune and Defer, 2001). Two isoforms, PKA-I and PKA-II, exist and they differ in regulatory subunits. PKA holoenzymes are inactive heterotetramers. Binding of two cAMP molecules to each of the regulatory subunits results in the release and activation of the catalytic subunits. These catalytic subunits will phosphorylate serine and threonine
residues on specific substrate both in the cytoplasm and in the nucleus (Skalhegg and Tasken, 2000). Although PKA are highly versatile, its specificity and signal fidelity is regulated by a complex of protein called A-kinase-anchoring proteins (AKAPs) that control both spatial and temporal dynamics of PKA (Beene and Scott, 2007; Michel and Scott, 2002; Tasken and Aandahl, 2004). AKAPs are bound to cytoskeletal proteins or organelles and bind regulatory subunits of the PKA, so that the PKA can be docked and concentrated in proximity to their targets and can phosphorylate only selected proteins (Gold et al., 2006) (Figure 9).

The major nuclear targets of PKA are the transcription factors of the cAMP response element binding (CREB) family (Mayr and Montminy, 2001). CREB proteins bind optimally to CREs sequence in promoters and upon phosphorylation by PKA they recruit the coactivator, CREB binding protein (CBP) to the promoter. Such a

Figure 9. Overview of PKA activation. Upon stimulation by ligands, G protein-coupled receptor (GPCR) induces activation of adenylyl cyclase (AC) through Gαs protein. A pool of cAMP is generated by AC in localized region within the cell. cAMP activates PKA by dissociating PKA from AKAP. From Altarejos and Montminy, 2011
phosphorylation event results in the induction of cellular gene expression (Sands and Palmer, 2008).

Several studies have linked PKA signaling pathway and different cancers (Naviglio et al., 2009). PKA play important roles in cell cycle progression. Low levels of cAMP are present during mitosis, while higher levels are present in G1 and early S; on the other hand, PKA phosphorylates macromolecular complexes responsible for the destruction of mitotic cyclins and separation of the sister chromatids during anaphase to metaphase transition (Ferrari, 2006). The PKA pathway has been reported to stimulate cell growth in many cell types while inhibiting some others (Insel et al., 2012; Stork and Schmitt, 2002). An involvement of PKA in neoplastic transformation and tumor growth, especially in the onset and maintenance of endocrine tumors (hormone-responsive tissues) has been demonstrated (Mantovani et al., 2008; Rivas and Santisteban, 2003). Overexpression of PKA-I isoform, as compared with the PKA-II one, is considered a hallmark of most human tumors, correlating with more severe pathological features in several tumor types (Cho-Chung and Nesterova, 2005; Tortora and Ciardiello, 2002). Furthermore, PKA catalytic β subunit has been shown to be a direct transcriptional target of c-MYC, and proposed as a crucial component of the program by which constitutive c-MYC expression contributes to cell transformation (Wu et al., 2002). Another function, in which PKA may contribute to dysregulation in cancer is the actin-based cell migration that involves cytoskeleton remodeling. PKA regulates actin dynamics, by targeting structural proteins, like actin, integrins, VASP and myosin light chain, and regulatory proteins, like Rho GTPases, Src kinases, PAKs, phosphatases and proteases (Howe,
The involvement of PKA in migration and invasion of breast carcinoma cells and ovarian cancer cells has been described (Jiang et al., 2009; McKenzie et al., 2011).

PKA has been associated with hormone therapy resistance in breast cancer. Serine 305 on ERα is a known target of PKA. Serine 305 resides at the C-terminus of the hinge region that provides a centre of rotation to the total ERα. The region around serine 305 is a multifunctional domain that binds to many coregulatory proteins and is involved in the regulation of activity and stability of ERα (Barone et al., 2010). Phosphorylation of Ser 305 occurs by protein kinase A and is associated with resistance to tamoxifen in patients (Bostner et al., 2010; Holm et al., 2009a; Kok et al., 2011). Phosphorylation of serine 305 by PKA, keeps ERα in an active conformation when tamoxifen is bound, which means that it mimics an estrogen-bound ERα (Michalides et al., 2004); therefore, understanding the mechanisms underlying PKA regulation of ERα is crucial.
I. MATERIALS AND METHODS

Cells—MCF-7 clones stably overexpressing vector, HA-tagged PAK1 WT and PAK1 Y3F and SKBR3 cells were maintained in DMEM (Corning Cellgro) supplemented with 10% FBS (Sigma-Aldrich). T47D and TMX2-28 cells stably overexpressing GFP, myc-tagged PAK1 WT and PAK1 Y3F were maintained in RPMI (Corning Cellgro) supplemented with 10% FBS (Sigma-Aldrich) (Hammer et al., 2013; Rider et al., 2013a).

Plasmids—pRev-TRE-HA-PAK1 WT and pRev-TRE-HA-PAK1 Y3F plasmids were constructed as follows. The cDNAs encoding hemagglutinin (HA) epitope-tagged PAK1 WT and PAK1 Y3F were excised from pCMV6 constructs (described in (Rider et al., 2007)) and ligated into pRev-TRE (obtained from Dr. Thomas, Vollum Institute, Portland, OR). MCF-7 cells were transfected with pRevTet-ON (obtained from Dr. Thomas, Vollum Institute, Portland, OR) and stable clones were selected with G418 (1 mg/ml). A chosen positive Tet-ON clone was then transfected with pRev-TRE-HA-PAK1 WT, pRev-TRE-HA-PAK1 Y3F or pRev-TRE alone. Stable cells were selected with hygromycin B (0.5 mg/ml) and expressing clones were isolated. Inducible protein expression was assayed by Western blotting with αHA Ab. Stable clones were maintained with G418 (0.5 mg/ml) and hygromycin B (0.1 mg/ml). These clones express HA-tagged PAK1 WT or PAK1 Y3F when cells were treated with 1 µg/ml doxycycline for at least 24 h. The cDNA constructs encoding myc-tagged PAK1 WT and PAK1 Y3F were described earlier (Hammer et al., 2013). cDNA construct encoding wild type Etk and kinase-dead Etk KQ in pcDNA3 vector were described in [25]. cDNA encoding
βPIX WT, βPIX DHm(L238R/L239S), which lacks GEF activity, and βPIX SH3m(W43K), which is PAK1-binding-deficient were described in [39]. cDNAs encoding Gβ1-mCherry, Gβ2-Venus, Gγ2-YFP, Gγ5-YFP and Gγ13-YFP were purchased from Addgene.

Construction of PAK1 WT in the pLNCX2 retroviral vector containing the IRES2-EGFP element was described previously (Li et al., 2008). Tyrosines 153, 201, and 285 in PAK1 WT were mutated to phenylalanines using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, California) (PAK1 Y3F). Mutations were confirmed by sequencing by the University of Michigan DNA Sequencing Core. The final plasmids PAK1 WT and PAK1 Y3F with N-terminal myc-tags were expressed from retroviral constructs that include IRES elements that allow the transcription of a single bicistronic mRNA of myc-PAK1-IRES2-EGFP, and therefore produce myc-PAK1 together with EGFP as a reporter for expression of PAK1. PAK1 pSUPER-GFP that targets the PAK1 mRNA and the mutated control PAK1 pSUPER-GFP were described early (Li et al., 2003b). Monoclonal anti-myc (9E10) antibody (Ab), polyclonal anti-IκB-α, and polyclonal anti-PAK1 Ab (N-20) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Polyclonal anti-phospho-ERK5 (Thr218/Tyr220), and polyclonal anti-ERK5 Abs were from Cell Signaling Technology, Inc. (Danvers, Massachusetts). Polyclonal anti-MMP-1 and polyclonal anti-MMP-3 Abs were from Triple Point Biologics, Inc. (Forest Grove, Oregon), polyclonal anti-MMP-2 (ab37150) was from Abcam (Cambridge, Massachusetts), and polyclonal anti-MMP-9 (AB13458) was from EMD Millipore (Billerica, Massachusetts). Monoclonal antiactin Ab (pan Ab-5, clone ACTN05) was from Thermo Scientific (Rockford, Illinois). Monoclonal anti-HA Ab was
from Roche Applied Science (Indianapolis, Indiana). Monoclonal anti-γ-tubulin Ab was from Sigma-Aldrich (St. Louis, Missouri). Human PRL was purchased from the National Hormone and Peptide Program (Dr. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland). TNF-α was from Invitrogen (Carlsbad, California).

Immunoprecipitation and immunoblotting—Cells were serum deprived for 48h and treated with ligands with indicated concentrations for indicated times. The cells were rinsed 3 times with 10mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM Na orthovanadate. Cells were then solubilized in lysis buffer (50 mM Tris, pH 7.5, 0.1% Triton X-100, 150 mM NaCl, 2 mM EGTA, 1 mM Na orthovanadate, 1 mM phenylmethysulfonyl fluoride, 10 µg/mL aprotonin, 10 µg/mL leupeptin) and centrifuged at 14,000 × g for 10 minutes at 4°C. The supernatant (cell lysate) was boiled for 5 minutes in a SDS-PAGE sample buffer (250mMTris-HCl, pH 6.8, 10% sodium dodecyl sulfate, 10% β-mercaptoethanol, 40% glycerol, 0.01% bromphenol blue). For immunoprecipitation HA-PAK1 was immunoprecipitated (IP’ed) from the cell lysate using αHA and protein A-agarose. The solubilized proteins were separated by SDS-PAGE followed by immunoblotting with the indicated antibodies and visualization with the enhanced chemiluminescence detection system. All immunoblots presented in the same figure derive from the same gel. All blots are representative of at least three experiments.

Antibodies, siRNA and reagents—Primary Abs used in this study were polyclonal αBmx/Etk (H-220), monoclonal αmyc Ab (9E10), polyclonal αGPR30 (GPER1; N-15), polyclonal αGβ (T-20), polyclonal αERα (HC-20) and polyclonal αIkB-
α from Santa Cruz Biotechnology, Inc.; monoclonal αHA were from Covance; monoclonal αPY (clone 4G10) were from EMD Millipore; monoclonal αtubulin were from Sigma-Aldrich, monoclonal αGFP were from Thermo Fisher Scientific. polyclonal αphospho-ERα (Ser305) (16J4) were from Bethyl Lab., Inc. Rabbit monoclonal αJAK2, polyclonal αPAK1, polyclonal αpPAK1(Thr423)/PAK2(Thr402), polyclonal αβPIX, rabbit monoclonal αphospho-p38 (Thr180/Tyr182), rabbit monoclonal αp38 MAPK, mouse monoclonal αphospho-SAPK/JNK (Thr183/Tyr185) (G9), rabbit monoclonal αSAPK/JNK (56G8), monoclonal αphospho-p44/42 MAPK (Thr202/Tyr204), polyclonal αp44/42 MAPK, rabbit monoclonal αSrc (32G6), monoclonal αphospho-ERα (Ser118) (16J4), monoclonal αphospho-Akt (Ser473) (D9E) and polyclonal αAkt were from Cell Signaling Technology, Inc. Etk/Bmx, GPER1, ERα, βPIX siRNAs were from Santa-Cruz Biotechnology, Inc.; PAK1 and negative control nontargeting siRNA were purchased from Cell Signaling, Inc. 17β-estradiol (E2) was purchased from Sigma-Aldrich, human PRL was from the National Hormone and Peptide Program (Dr. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD), HRG was from Thermo Scientific, and TNF-α from Invitrogen. Inhibitors SB203580, SP600125, IKK inhibitor VII, AG 879, LY294002, PP1 and PP2 were from EMD Chemicals, Inc.; U0126 was from Cell Signaling, Inc.; myristilated-PKI amide (14-22) and IPA3 were from Tocris Biosciences, H-89 was from Cayman Chemicals, Rp-cAMP from Santa-Cruz Biotechnology, Inc.; 4-cyano-3 methylisoquinoline (4C3MQ) was from Toronto Research Chemicals, Inc.; Canertinib and STI 571 were from Sigma-Aldrich. [γ-32P]ATP was from MP Biomedic, histone H4 was from New England Biolabs, kemptide was from Sigma-Aldrich.
DIF derivatives (structures depicted in Figure 10) were synthesized as described previously (Gokan et al., 2005). Epidermal growth factor (EGF) and estrogen (E2) were purchased from BD Biosciences and Sigma-Aldrich, respectively. Polyclonal αPAK (N-20; Santa Cruz Biotechnology), rabbit monoclonal αJAK2 (Cell Signaling) antibodies were used in this study.

PAK1 in vitro kinase assay—To assess PAK1 WT and PAK1 Y3F in vitro kinase activity, HA-PAK1 were immunoprecipitated (IPed) with α-HA antibody from cells treated with or without PRL (200 ng/ml, 20 min), E2 1 nM, 30 min, HRG 30 ng/ml, 15 min and subjected to an in vitro kinase assay in kinase buffer (50 mM HEPES, 100 mM NaCl, 5 mM MnCl2, 0.5 mM dithiothreitol, 1 mM Na3VO4; pH 7.6) in the presence of 10 μCi [γ-32P] ATP (MP Biomedical, Santa Ana, CA, USA), and 5 μg of histone H4 (substrate of PAK1; New England Biolabs, Ipswich, MA) 10 μg/ml aprotinin, and 10 μg/ml leupeptin at 30° C for 30 min. Relative levels of incorporation of 32P into histone H4, an indicator of phosphorylation, were assessed by autoradiography and estimated by a phosphoimager screen. The same membrane was blotted with αPAK1 to assess the
amount of PAK1 for each condition. Nitrocellulose patterns were scanned and the amount of PAK1 was quantified using Multi-Analyst (Bio-Rad Laboratories, Hercules, CA, USA) software. Relative PAK1 kinase activity was then normalized by the amount of IPed PAK1 for each lane.

PKA kinase assay—PKA kinase assay was performed as previously described (Corbin and Reimann, 1974). Briefly, to assess PKA kinase activity, E2 treated cells were washed with cold PBS and and scraped into kinase buffer (50 mM HEPES, 100 mM NaCl, 5 mM MnCl2, 0.5mM dithiothreitol, 1 mM Na3VO4; pH 7.6, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Cells were lysed by 5 freeze/thaw cycles with 10 sec bursts of vortexing between cycles. Cell lysate was cleared by spinning at 13000 × g at 4º C for 10 min. Equal amount of protein was subjected to an in vitro kinase assay in the presence of 5 µCi [γ-32P] ATP (MP Biomedical), and 3 µg kemptide (synthetic peptide substrate for PKA: Sigma-Aldrich) at 30º C for 10 min. 20µl of the reaction volume was spotted on P81 phosphocellulose paper (EMD Millipore), washed three times in large volumes of 1% phosphoric acid and once in acetone, and let air-dry. The phosphocellulose paper was subjected to liquid scintillation counting in Beckman LS 6000IC (Beckman Coulter, Fullerton, CA, USA).

To assess direct inhibition of PAK1 by DIF-3(+1), GST- tagged PAK1 were purified using a glutathione-agarose affinity columns (Sigma-Aldrich). The purity of the proteins was monitored by SDS-PAGE. GST-tagged PAK1 was incubated with DIF-3(+1) or THPH at 30ºC for 30 min in kinase buffer containing 10 µCi of [γ-32P] ATP, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Proteins were separated by SDS-PAGE and visualized by autoradiography followed by immunoblotting with indicated Ab.
Treatment with inhibitors—MCF7 PAK1 WT cells were serum deprived for 48h, and treated with E2 (1nM, 30min) in the presence or absence of inhibitors: LY294002 (10µM, 1h); U0126 (10µM, 1h); SB203580 (10µM, 6h); SP600125 (30µM, 1h); AG879 (increasing concentrations, 1h); PP1 or PP2, (increasing concentrations, 2h ); GF109203X (5µM, 2h); IKK inhibitor VII (5µM, 16h); imatinib mesylate (STI 571; increased concentrations, 24h); or canertinib (CNT, increased concentrations, 4h). To inhibit PKA, H89 (10µM, 1h); myristolated-PKI amide (14-22) (myr; 20µM, 1h), Rp-cAMP (100µM, 2h), and 4-cyano-3 methylisoquinoline (4C3MQ; 2µM, 4h) were used. Whole cell lysates were probed with indicated Ab. For ETK and Src inhibition, ETK and Src were IP’ed with the αETK and αSrc respectively and subjected to in vitro kinase assay. Autophosphorylation was assessed by incorporation of [γ-32P]ATP . The same membranes were blotted with αETK and αSrc respectively.

TMX2–28 clones were treated with inhibitors before and during incubation with PRL. Cell viability was monitored by trypan blue exclusion. For treatment with SB203580 (EMD Chemicals, Inc.), cells were deprived for 24 hours, treated with 10 µM SB203580 for 6 hours prior to plating the cells with or without PRL for 48 hours in the presence of SB203580 (final concentration, 10 µM). For treatment with JNK Inhibitor II (SP600125) (EMD Chemicals, Inc.), cells were deprived for 24 hours, treated with inhibitor (30 µM) for 1 hour prior to plating with or without PRL for 48 hours in the presence of SP600125 (final concentration, 15 µM). For treatment with U0126 (Cell Signaling, Inc.), cells were deprived for 24 hours and treated with the inhibitor (10 µM) for 1 hour prior to plating with or without PRL for 48 hours in the presence of U0126 (final concentration, 10 µM). For MMP inhibition, TMX2–28 clones were treated with
MMP-2 inhibitor I (125 µM; overnight) (Millipore Corp., Bedford, Massachusetts), MMP-3 inhibitor I (50 µM; overnight) (Alpha Diagnostic International, San Antonio, Texas), or MMP-9 inhibitor I (50 µM; overnight) (Millipore). Cells were allowed to invade for 48 hours in the presence of the MMP inhibitors.

Gene Silencing—For siRNA transfection, specific siRNAs (PAK1 and βPIX (Santa-Cruz), Etk/Bmx, GPER1, ERα (Cell Signaling)) or negative control nontargeting siRNA (Cell Signaling) were transiently transfected using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. The final concentration of siRNA was 100 nM. In siRNA rescue experiments, 24h after siRNA transfection, cells were transiently transfected with cDNA using a modification of the polyethylenimine method (Hammer et al., 2015a). Briefly, the cDNA was added to plain DMEM, followed by addition of PEI at a ratio of 3:1 (µg PEI: µg cDNA), and the mixture was then incubated for 10 minutes at room temperature prior to addition to cells. Cells were incubated at 37 °C for 48 hours. Knock-down and re-expression were confirmed by Western blot analysis.

For synthesis of PAK1 siRNA in vivo, TMX2–28 cells were transiently transfected with cDNA encoding PAK1 pSUPER-GFP that targets the PAK1 mRNA or control pSUPER-GFP that produces a siRNA that is 2 bp different from the PAK1 siRNA using Nucleofector kit V (Amaxa Biosystems, Gaithersburg, Maryland) according to the manufacturer’s protocol. MMP-1 was knocked down by using short hairpin RNA (shRNA) expression construct as described previously (Beshir et al., 2010; Gupta et al., 2007). Briefly, MMP-1 shRNA expression vector was cotransfected with a pVSV-G vector into the GPG29 amphotropic packaging cell line using a modification of the
polyethylenimine method (Boussif et al., 1995). In 48 hours the virus broth was collected and added to the TMX2–28 clones. MMP-1 knockdown was confirmed by Western blot analysis with anti-MMP-1 in 48 hours after infection.

Cell proliferation assay— MCF7 clones (5×10³ cells/well) and T47D clones (5×10³ cells/well) were seeded on 96 well plate and allowed to adhere for 24 h. Cells were serum deprived for 24 h in phenol red-free DMEM supplemented with 1% charcoal-stripped serum. Cells were allowed to proliferate for 7 days in the corresponding treatment conditions: each well contained 200 µl of DMEM supplemented with 2% charcoal-stripped serum treated with or without 200 ng/ml of PRL and 1 nM E2. Treatment conditions were replaced every 24 h. Cell proliferation was assessed by MTT cell proliferation assay according to manufacturer’s protocol. Briefly, the medium on cells was replaced with 100 µl plain medium. 10 µl of 12 mM MTT solution was added to each well and the plate was incubated at 37°C for 4 h. 100µl of 10% solution of SDS in 0.01M HCl was added to each well and mixed thoroughly. The plate was incubated at 37°C overnight (15 h) in a humidified chamber and absorbance at 570 nM was read in SpectraMax M5 (MDS Analytical Technologies, Sunnyvale, CA). Data from 3 wells were polled and plotted as percentage proliferation from untreated control. Each experiment was repeated at least 3 times. For the inhibition studies, cell proliferation assays were performed as described above with the addition of IPA3 with the indicated concentration.

For assessment of the effect of DIF-3 (+1) on MCF-7 and T47D cells stably expressing PAK1 WT (5×10³ cells/well) were allowed to grow for 7 days in 96-well plates. Each well contained 200 µl of DMEM (for MCF-7 clone) or RPMI (for T47D
clone) were treated with either vehicle, 500 ng/ml of PRL, 1nM E2, 30 ng/ml HRG or 10 ng/ml EGF with or without 5µM DIF-3(+1). Cell proliferation was assessed by MTT cell proliferation assay as described above.

Luciferase reporter gene assay—Luciferase assay was performed as described previously (Tao et al., 2011). MCF-7 cells stably overexpressing PAK1 WT were co-transfected with cyclin D1-luciferase reporter and pCH110 plasmid containing a functional lacZ gene. The cells were serum deprived for 24 hrs, treated with either vehicle, 200 ng/ml of PRL, 1nM E2, 30 ng/ml HRG or 10 ng/ml EGF with or without 5 µM DIF-3(+1) for an additional 24 h. The cells were lysed, and luciferase activity was measured using Luciferase assay kit (Promega) according to the manufacturer’s protocol. Luciferase values were corrected for transfection efficiency by determining the ratio of luciferase activity to β-galactosidase activity and expressed as “normalized cyclin D1-luciferase activity.” Each transfection was performed in triplicate wells.

Cell invasion assay—Equal numbers of deprived TMX2–28 cells (5 × 10⁵ cells) were placed in deprivation media (DM; 2% BSA) in the upper chamber of a Boyden chamber (Corning, Inc.) coated with Matrigel (BD Biosciences, Palo Alto, California). DM with or without 100 ng/mL hPRL was placed in the lower chamber. After 48 hours cells from 5 separate fields that had invaded the Matrigel were counted after fixation and staining with 4’,6-diamidino-2-phenylindole.

Preparation of Collagen gels—For cells plated on Collagen I (BD Biosciences) or Collagen IV (Sigma), plates were coated with rat tail type I Collagen or human placental Collagen IV (10µg/cm²). For embedded growth in Collagens, 0.5 ×10⁶ cells in 2 mg/mL,
Collagen was applied to 24-well plates and DM, with or without 100 ng/mL PRL, was added. After 48 hours conditioned medium was collected and processed for ELISA.

ELISA—After incubation for with 48 hours with or without 100 ng/mL PRL in plain DMEM, immunoreactive MMP-1, -2, -3, and -9 in conditioned medium were quantified by ELISA (R&D Systems, Minneapolis, Minnesota). MMP-1, MMP-2, MMP-3, and MMP-9 assays recognize total (pro- and active) MMP-1, -2, -3, and -9 forms, respectively. 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Pittsburgh, Pennsylvania; Pierce Chemical Co., Rockford, Illinois) was used for all ELISA experiments. Conditioned medium was concentrated prior to ELISA.

Statistical Analysis—Data from at least 3 separate experiments were pooled and analyzed using 1-way analysis of variance plus Tukey’s honest significant difference test. Differences were considered to be statistically significant at a P < 0.05. Results are expressed as the mean ± standard error (S.E.).
II. RESULTS

A. The role of tyrosyl phosphorylated PAK1 in the synergetic effect of estrogen and prolactin in breast cancer cells

The involvement of estrogen in the development of cancer has been known for years; however, the exact mechanisms responsible are far from clear. The participation of prolactin in the pathogenesis of breast cancer is increasingly appreciated. 80-90% of breast carcinomas express the PRL receptor (PRLR; (Touraine et al., 1998)) while 75% breast carcinomas express ERα (Karayiannakis AJ, 1996) resulting in the majority of breast tumors expressing both receptors. Even though PRL and estrogen exert direct and independent effects on breast cancer cells, there is crosstalk between the two hormones. This crosstalk occurs at multiple levels and is bidirectional in that modulation of the estrogen pathway influences PRL pathways and vice versa. Such crosstalk can lead to enhanced estrogen-induced proliferation of breast cancer cells by PRL (Gutzman et al., 2005). In the present study, we sought to elucidate the mechanism by which estrogen and prolactin activate estrogen receptor and a role of PAK1 in this process.

17β-estradiol (E2) is a well-known hormone responsible for proliferation of normal and neoplastic breast tissue. Because PAK1 also stimulates cell proliferation (Eswaran et al., 2012), we tested whether overexpression of PAK1 WT enhances proliferation in MCF-7 and T47D breast cancer cells in response to E2. As demonstrated previously, E2 induced proliferation of control T47D and MCF-7 cells (Figure. 11, upper plots). Stable overexpression of PAK1 WT did not affect basal cell proliferation while E2 stimulated cell growth in both cell lines (Figure. 11, PAK1 WT cells). In contrast, stable
overexpression of PAK1 Y3F (phospho-tyrosyl-deficient PAK1 Y3F mutant in which 3 JAK2 phosphorylation sites are mutated) strongly decreased E2-dependent cell proliferation (Figure. 11, PAK1 Y3F cells). To confirm the role of PAK1 in cell proliferation, we treated control MCF-7 and T47D cells with cell-permeable small-molecule PAK1 inhibitor IPA-3 (Deacon et al., 2008). IPA-3 did not inhibit proliferation of vehicle-treated cells and was not toxic but it did inhibit E2-dependent cell proliferation.
in a concentration-dependent manner (Figure 11, bottom plots) confirming that PAK1 participates in E2-dependent cell proliferation.

To start uncovering the mechanism by which pTyr-PAK1 regulates E2-dependent cell proliferation, we assessed PAK1 kinase activity in E2-treated cells, because E2 activates PAK1 (Mazumdar and Kumar, 2003a). As demonstrated previously, PRL activated PAK1 WT significantly stronger than PAK1 Y3F confirming that tyrosines 153, 201 and 285 are required for full activation (Figure 12, upper panel; (Hammer et al., 2013; Rider et al., 2013a)). Activation of PAK1 WT by E2 was similar to the activation by PRL. Surprisingly, E2 failed to activate PAK1 Y3F (Figure 12, lower panel).

**Figure 12.** Estrogen activates PAK1 WT but not PAK1 Y3F. (B) PAK1 WT or PAK1 Y3F clones of MCF-7 cells were treated with PRL (top), or E2 (bottom). PAK1 was IP’ed, subjected to an *in vitro* kinase assay with H4 histone as a substrate and probed with αHA for HA-PAK1. Relative PAK1 kinase activity was normalized by IP’ed PAK1 for each lane and plotted. *, p <0.05 compared with control cells in the same condition, n=3
Additionally, E2-induced phosphorylation of Thr423-PAK1 (a marker of PAK1 activation) was detected in PAK1 WT but not in PAK1 Y3F cells (Figure 13) demonstrating that PAK1 tyrosyl phosphorylation is important for E2-dependent PAK1 activation. To confirm that PAK1 Y3F retains its kinase activity, we assessed effect of the heregulin (HRG), a known activator of PAK1 (Adam et al., 1998), on PAK1 activation. HRG-induced PAK1 kinase activity was similar in cells expressing PAK1 WT and PAK1 Y3F (Figure 13) confirming that PAK1 Y3F is functionally active.

In attempt to understand how E2-mediated PAK1 tyrosyl phosphorylation and activation, we first tested whether E2 activates JAK2. As expected, PRL strongly activated JAK2 (Figure 14A, lane 6) but E2 did not (Figure 14A, lanes 1-5). We hypothesized that kinase(s) other than JAK2 tyrosyl phosphorylated PAK1 in response to

![Figure 14A](image)

(A) PAK1 WT cells were treated either with E2 for indicated times or with PRL for 30 min. JAK2 was IP'ed and probed for tyrosyl phosphorylation and re-probed for total JAK2.

(B) PAK1 WT clone was treated with indicated concentrations of c-Abl inhibitor STI or with ErbB pan inhibitor CNT prior to E2 treatment. IP'ed PAK1 and whole cell lysate (WCL) were probed with indicated antibodies.

(C) PAK1 WT clone was treated with indicated concentration of PP1 or PP2. IP'ed PAK1 and whole cell lysate (WCL) were probed with indicated antibodies (C, left panel). Src was IP'ed and subjected to an in vitro kinase assay (C, right panel).
E2, and tested tyrosine kinases Etk/Bmx, Src, c-Abl and ErbB because they have been previously implicated in tyrosyl phosphorylation of either PAK1 or PAK2 (Bagheri-Yarmand et al., 2001; McManus et al., 2000; Renkema et al., 2002; Roig et al., 2000). Inhibition of c-Abl by STI571 and ErbB by canertinib (CNT) did not affect tyrosyl phosphorylation of PAK1 in response to E2, although activation of Erk 1/2 was eliminated by both inhibitors (Figure 14B). Src inhibitors PP1 and PP2 did not affect tyrosyl phosphorylation of PAK1 in response to E2 but did block Src activation as assessed by Src in vitro kinase assay (Figure 14C). Inhibition of Etk by AG879 resulted in a marked, concentration-dependent decrease in pTyr-PAK1 signal (Figure 15A, left) suggesting that Etk is involved in E2-dependent PAK1 phosphorylation. Tyrosines 153, 201 and 285 are implicated in this E2-dependent phosphorylation because Y3F failed to be phosphorylated in response not only to PRL but also to E2 (Figure 15A, right). More importantly, inhibition of Etk attenuated PAK1 kinase activity in response to E2 (Figure 15B). To support this finding, we knocked-down Etk by siRNA and assessed PAK1 kinase activity in response to E2. Indeed, silencing of Etk decreased PAK1 activity in response to E2 (Figure 15C). We were able to recover E2-dependent activation of PAK1 by re-introducing WT, but not kinase-dead Etk, in the Etk-depleted cells (Figure 15C; (Qiu et al., 1998a)). These data strongly suggest that Etk tyrosyl phosphorylates and activates PAK1 in response to E2.
Figure 15. Etk phosphorylates and activates PAK1 in response to estrogen. (A) PAK1 WT cells were treated with indicated concentration of Etk inhibitor AG879 before E2 treatment. IP’ed PAK1 and whole cell lysate (WCL) were probed with indicated antibodies. PAK1 or JAK2 were IP’ed from PAK1 WT and PAK1 Y3F clones treated with E2 or PRL and immunblotted with the indicated Abs (right panel). (B) PAK1 WT cells were treated with AG879. PAK1 was IP’ed, subjected to an in vitro kinase assay with H4 histone as a substrate and probed with αHA for HA-PAK1. Relative PAK1 kinase activity was normalized by IP’ed PAK1 for each lane and plotted. (C) The cells were transfected with control (scr) or Etk siRNA. In siRNA rescue experiments, 24h after Etk siRNA transfection, cells were transfected with either Etk WT or Etk kinase-dead mutant (KD). Silencing efficiency was judged by immunoblotting with αEtk. PAK1 kinase activity was assessed as in B *, p <0.05 compared with control cells in the same condition, n=3
To test whether mERα is required for E2-triggered PAK1 activation, we used TMX2-28 cells, an ER-negative variant of MCF-7 cells (Fasco et al., 2003). As expected, E2 caused phosphorylation of Ser118 and Ser305 of ERα (markers of ER activation) in MCF-7 cells but not in TMX2-28 cells (Figure 16A). Furthermore, TMX2-28 cells are also GPER1-negative (Figure 16A). E2 activated PAK1 in MCF-7 cells but not in TMX2-28 cells while PRL activated PAK1 similarly in both cell lines (Figure 16B) suggesting that either mERα or GPER1 are required for activation of PAK1 by E2.

Figure 16. Both GPER1 and mERα are involved in PAK1 activation in response to estrogen. MCF-7 and TMX2-28 cells were treated with E2 and either WCL were probed with indicated Abs (A) or in vitro kinase assay was performed as in Fig.12 (B). (C-D) MCF-7 and SKBR3 cells were treated with E2 and either WCL were probed with the indicated Abs (C) or in vitro kinase assay was performed as in Fig.12 (D).
To determine whether E2 activates PAK1 via GPER1, we employed ER-negative but GPER1-positive SKBR3 cells. GPER1 was activated by E2 in both MCF-7 and SKBR3 cells as demonstrated by Erk 1/2 phosphorylation while ERα was activated by E2 only in MCF-7 cells (Figure 16C). E2 activated PAK1 to a similar extent in both cell lines suggesting that GPER1 participates in PAK1 activation (Figure 16D). To confirm this observation, we performed knock-down of either GPER1, ERα or both receptors by corresponding siRNAs in MCF-7 cells. Silencing of either ERα or GPER1 significantly decreased E2-triggered PAK1 activation, however, only downregulation of the ERα and GPER1 together completely abolished PAK1 activation in response to E2 but not HRG (Figure 17). These data suggest that E2 promotes PAK1 activation through both mERα and GPER1 plasma membrane receptors.

Figure 17. Both GPER1 and mERα mediate PAK1 activation in response to estrogen. MCF-7 PAK1 WT cells were transfected with control (scr), ERα, GPER1 or ERα+GPER1 (ER/G) siRNA. HA-PAK1 was immunoprecipitated and subjected to in vitro kinase assay with H4 histone as a substrate and probed with αHA for HA-PAK1. Relative PAK1 kinase activity was normalized by IP’ed PAK1 for each lane and plotted. Bars represent mean ±S.E., *, p <0.05 compared with control cells in the same condition, n=3.
In order to determine which combination of β and γ G protein subunits participates in E2-dependent PAK1 activation, we overexpressed β1 or β2 together with γ2, γ5 or γ13 in different combinations and assessed E2-dependent PAK1 activity (Figure 18A). As expected, E2 activated PAK1 (lane 2 vs. lane 1). Overexpression of β1γ2 and β1γ5 strongly enhanced E2-dependent PAK1 activity (lanes 4 and 6). Interestingly, overexpression of β2γ2 (lane 8), β2γ5 (lane 10) and β1γ13 (lane 12) inhibited PAK1 activity in response to E2 while overexpression of β2γ13 (lane 14) did not change PAK1 activity as compared to the control (lane 2). To test whether 3 tyrosines of PAK1 play a role in this activation, we overexpressed β1γ2 and β1γ5 subunits in PAK1 WT and Y3F clones and assessed PAK1 activation in response to E2. Overexpression of β1γ5 increased both basal and E2-stimulated PAK1 WT activity (Figure 18B, lanes 5-6) while
overexpression of β1γ2 enhanced PAK1 WT activity only in response to E2 (lanes 3-4).

PAK1 Y3F mutant failed to be activated by β1γ2 and β1γ5 overexpression (lanes 9-12) whereas HRG strongly activated PAK1 WT and Y3F (lanes 13-14). To assess PAK1 binding to β1γ2 and β1γ5, PAK1 WT or Y3F were immunoprecipitated and assessed for associated β1γ2, β1γ5 (Figure 19A) and β1γ13 (Figure 19B). PAK1 WT bound to E2-activated β1γ2 and to β1γ5 independent of E2 (Figures 19A and 19B, lanes 3-6). We considered the binding to β1γ2 without E2 (Figures 19A and 19B, lane 3) and to β1γ13 (Figure 19B, lanes 1-2) as negligible. The same levels of negligible binding we demonstrated for PAK1 Y3F and β1γ13, β1γ2 and β1γ5 (Figures 19A and 19B).

Endogenous Etk was associated with E2-activated PAK1 WT (αEtk blot, lanes 2, 4 and 6 in Figures 19A and 19B) while PAK1 Y3F failed to bind to Etk (lanes 7-12). These data suggest that E2 treatment promotes formation of Gβ1γ2,5/PAK1/Etk complex.

**Figure 18.** Gβγ subunits of G protein activate PAK1 in response to estrogen. (A, B) Different combinations of β1, β2, γ2, γ5 and γ13 subunits were overexpressed. E2-dependent PAK1 activation was assessed in the *in vitro* kinase assay as in Fig. 12. Both gray bars in B represent the cells treated with HRG. Bars represent mean ±S.E., *, p <0.05 compared with control cells in the same condition, n=3.
Figure 19. E2-activated PAK1 makes a complex with Gβγ subunits of G protein, Etk and βPIX. (A and B). Different combinations of β1, β2, γ2, γ5 and γ13 subunits were overexpressed in MCF-7 clones. HA-PAK1 was IP’ed from PAK1 WT or Y3F clones and probed with the indicated antibodies. All blots are representative of at least 3 experiments.
To determine whether E2 alters βPIX-PAK1 interaction, we IP’ed PAK1 from PAK1 WT and Y3F cells treated with E2 over a time course and probed it for endogenous βPIX. E2 increased association of βPIX with PAK1 WT but not with Y3F (Figure 20A), demonstrating that tyrosyl phosphorylation of PAK1 facilitates its binding activity toward βPIX in response to E2. We next overexpressed βPIX in WT and Y3F clones and assess E2-dependent PAK1 kinase activity. βPIX significantly activated PAK1 WT in response to E2 as compared to PAK1 WT cells overexpressing vector and Y3F cells overexpressing βPIX (Figure 20B). βPIX siRNA-mediated knock-down was used to confirm the effect of βPIX on PAK1 in ERα activation (Figure 21). βPIX silencing abolished E2-dependent PAK1 activation (lane 3) while re-expression of βPIX WT increased it to the control level (lane 4). Re-expression of βPIX DHm(L238R/L239S), which lacks GEF activity, partially rescued PAK1 kinase activity (lane 5) and re-expression of βPIX SH3m(W43K), which lacks PAK1 binding activity, did not rescue PAK1 activation (lane 6).
Figure 20. βPIX facilitates E2-dependent PAK1 activation. (A) IP’ed PAK1 from WT and Y3F clones treated with E2 over a time course was assessed for endogenous βPIX. (B) βPIX was overexpressed in WT and Y3F clones and PAK1 kinase activity was assessed in the in vitro kinase assay as in Fig. 12. * indicates longer exposure revealing endogenous βPIX (lower double-arrow). Overexpressed βPIX is indicated by the upper double-arrow.
To test whether βPIX is a member of Gβ1γ2,5/PAK1/Etk complex, we IP'ed either WT or Y3F mutant from cells treated with or without E2 and blotted these immunoprecipitates for βPIX. βPIX was associated with E2-activated PAK1 WT (Figures 19A and 19B, αβPIX blot, lanes 2, 4, 6). PAK1 Y3F complexed with β1γ5 only, and independently of E2 (lanes 11-12). These data suggest that E2 induces formation of a multiprotein signaling complex containing pTyr-PAK1, Etk, β1/γ2 or β1/γ5 subunits along with βPIX. It appears that three tyrosines of PAK1 are required for the formation of this complex which facilitates PAK1 activation.

**Figure 21. βPIX regulate estrogen-dependent PAK1 activation.** PAK1 WT MCF7 clones were transfected with control (scr; lanes 1-2) or βPIX siRNA (lanes 3-4). In siRNA rescue experiments, βPIX WT(lane 4), DHm(L238R/L239S) (lane 5) or SH3m(W43K) (lane 6) mutants were re-expressed and PAK1 activation was assessed. HA-PAK1 was immunoprecipitated and subjected to *in vitro* kinase assay as in Figure 12. Bars represent mean ±S.E., *, p <0.05 compared.
E2 rapidly activates multiple signaling pathways, including MAPKs, PI3K, PKC, PKA, NFκB and Src (Levin, 2009). To determine which of these pathways are implicated in E2-triggered PAK1 activation, we treated MCF-7 cells with E2 and different selective inhibitors. We confirmed the specificity of these inhibitors in MCF-7 cells (Figure 22A).

**Figure 22.** Selective inhibitors inhibit multiple MAPKs, PI3K, PKC, NFκB and PKA pathways. (A) MCF-7 cells were incubated MAPKs, PI3K, PKC, NFκB inhibitors as indicated and (B) PKA inhibitors. Whole cell lysates were probed with the indicated antibodies.
Although all these drugs inhibited respective pathways, only PKA inhibition by H89 inhibited E2-dependent PAK1 kinase activity (Figure 23A). To confirm that this effect was specific to estrogen, we treated PAK1 WT cells with PRL in the presence of H89 and

**Figure 23. Reciprocal regulation of PAK1 and PKA in E2 signaling.** The cells were treated with different selective inhibitors: U0126 (ERK 1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK1/2 inhibitor), LY294002 (PI3K inhibitor); IKK inhibitor VI, GF109203X (PKC inhibitor) and H89 (PKA inhibitor). The cells were treated with E2 (A) or PRL (B) and PAK1 activation was assessed in the *in vitro* kinase assay as in Fig. 1. (C) PAK1 activation was assessed as in B in cells treated with different PKA inhibitors: H89, myristilated-PKI amide (14-22) (myr), Rp-cAMP and 4-cyano-3 methylisoquinoline (4C3MQ). The cells were treated with either vehicle, E2, or Forskolin (Fors). (D) Lysates of control, PAK1 WT and PAK1 Y3F cells were assessed for PKA activity in the *in vitro* kinase assay.
showed that PAK1 kinase activity was unaffected (Figure 23B). In addition to H89, we tested other selective PKA inhibitors, myristolated-PKI amide (14-22) (myr), Rp-cAMP and 4-cyano-3 methylisoquinoline (4C3MQ) (Figure 22B) and found that all inhibited E2-activated PAK1 (Figure 23C). Furthermore, treatment of the cells with Forskolin, which triggers general cAMP elevation and PKA activation, enhanced PAK1 activity while Rp-cAMP abolished it (Figure 23C). In an attempt to determine whether the regulatory relationship between PKA and PAK1 is reciprocal, we hypothesized that activated PAK1 may in turn affect PKA activity. We observed significant elevation of PKA in in vitro kinase activity in E2-treated, but not in RPL-treated, PAK1 WT cells (Figure 23D). Interestingly, PAK1 Y3F inhibited E2-triggered activation of PKA (Figure 23D). These data suggest that, in response to E2, activated pTyr-PAK1 potentiates activation of PKA. At the same time, PKA is required for full PAK1 activation in response to E2, demonstrating a regulatory loop between these two kinases in E2 signaling.

Serine 305 of ERα can be phosphorylated by PAK1 and PKA (Michalides et al., 2004; Wang et al., 2002). Constitutively active PAK1 phosphorylates this serine resulting in subsequent phosphorylation of Ser118-ERα, ligand-independent ER activation and tamoxifen resistance (Rayala et al., 2006b; Wang et al., 2002). Overexpression of PAK1 WT strongly increased phosphorylation of both Ser305 and Ser118 of ERα upon E2 treatment (Fig. 24A, lanes 3 vs. 7, white and black bars) while overexpression of Y3F inhibited pSer118-ERα signalling (Figure 24A, lanes 11 vs. 3). PRL treatment induced Ser305- ERα phosphorylation, but not Ser118-ERα phosphorylation in WT PAK1 cells while Y3F cells demonstrated no PRL effect (Figure 24A, lanes 2, 6 and 10). Next we
Figure 24. Ser305-ERα is phosphorylated by PAK1 in response to PRL. (A) Control, PAK1 WT or PAK1 Y3F clones of MCF-7 cells were treated with vehicle, E2, PRL or PRL+E2 and WCL were immunoblotted for the indicated Abs. The graph represents the densitometric analysis of the bands obtained for pSer305-ERα and pSer118-ERα normalized with total ERα for each lane. Fold induction compared with the untreated control was plotted for three independent experiments. (B) The cells were transfected with control (scr) or PAK1 siRNA, treated with PRL, E2 or PRL+E2 and WCL were immunoblotted for the indicated Abs. (C) In siRNA rescue experiments, 24h after PAK1 siRNA transfection, cells were transfected with control or PAK1 WT, treated with PRL and WCL were immunoblotted with indicated Abs.
aimed to determine whether PAK1 or PKA participate in E2- and PRL-triggered ERα phosphorylation. We observed that PAK1 silencing with siRNA abolished PRL-dependent Ser305-ERα phosphorylation while E2-dependent pSer305-ERα signal was unaffected (Figure 24B, lanes 2 vs. 4). To confirm PAK1 is the kinase that phosphorylates Ser305-ERα in response to PRL, we re-introduced PAK1 in PAK1-silenced cells and demonstrated recovery of PRL-dependent Ser305-ERα phosphorylation (Figure 24C). To test whether PKA phosphorylates Ser305-ERα in response to E2, we inhibited PKA by Rp-cAMPs, and observed that pSer305-ERα signal was strongly decreased in the cells treated with E2 or Foskolin (Figure 25A, lanes 3 vs. 8 and 5 vs. 10) but not in the cells treated with PRL or PRL+E2 (Figure 25A, lanes 2 vs. 7 and 4 vs. 9). These data suggest that PKA phosphorylates Ser305-ERα in response to E2 while PAK1 phosphorylates Ser305-ERα in response to PRL. PAK1 augments PKA-dependent Ser305-ERα phosphorylation, as overexpression of PAK1 WT significantly increased pSer305-ERα signal in response to E2 as compared to the control cells (Figure 24A, lines 7 vs. 3). Furthermore, tyrosines 153, 201 and 285 of PAK1 participate in both signaling pathways because overexpression of Y3F mutant inhibited pSer305-ERα signal for both PRL and E2 treatments (Figure 24A, lanes 10-12). Since PAK1 WT, but not Y3F, significantly enhanced E2-dependent activation of PKA (Figure 23D), our data suggest that although PAK1 does not phosphorylate Ser305-ERα upon E2 treatment, it augments PKA activation and subsequent Ser305-ERα phosphorylation, thereby indirectly increases estrogen responsiveness.

If both PKA and PAK1 phosphorylate the same site on ERα, we hypothesized that PRL+E2 treatment would have additive effects on Ser305-ERα phosphorylation and
subsequent elevated Ser118-ERα phosphorylation. Indeed, we observed amplified pSer305-ERα and pSer118-ERα when PAK1 WT cells were treated with PRL+E2 as compared to the same cells treated either with PRL or E2 alone (Figure 24A, lanes 8 vs. 7 and 6). S305-ERα phosphorylation by PAK1 promotes ERα transactivation, thereby leading to up-regulation of cyclin D1 and hormone independence (Balasenthil et al., 2004a). To determine whether enhanced ERα activation had a physiological impact on breast cancer cells, we evaluated the effect of PRL+E2 on cell proliferation. In agreement

Figure 25. Ser305-ERα is phosphorylated by PKA in response to E2 and by PAK1 in response to PRL. (A) The cells were incubated with either vehicle or Rp-cAMP, treated with vehicle, PRL, E2, PRL+E2 or Forskolin(Fskl). Whole cell lysates were immuno-blotted with indicated antibodies. (B) MCF-7 and T47D cells overexpressing vector, PAK1 WT or PAK1 Y3F were incubated with vehicle, PRL, E2 or PRL+E2 and assessed for cell proliferation in 7 days. Changes in cell numbers at day 7 are shown as percentages of the vehicle-treated cell number. Bars represent mean ± S.E., *, p < 0.05 compared with control cells in the same condition, n=3.
with previously published studies demonstrating that PRL enhances E2-induced proliferation of breast cancer cells (Chen et al., 2010; Gonzalez et al., 2009; Gutzman et al., 2005; Rasmussen et al., 2010b; Sato et al., 2013), PRL+E2 induced proliferation of control T47D and MCF-7 cells (Figure 25B, control cells). Overexpression of PAK1 WT further enhanced cell proliferation in response to E2 +PRL as compared to the same cells treated with either PRL or E2 (Figure 25B, PAK1 WT cells). In contrast, stable overexpression of PAK1 Y3F mutant inhibited E2+PRL-dependent cell proliferation in T47D and MCF-7 clones (PAK1 Y3F cells) implicating tyrosines 153, 201 and 285 of PAK1 in regulation of PAK1 activities.

**Discussion**

PAK1 is a serine/threonine kinase and is involved often in cytoplasmic signaling cascades. Here we sought to determine the role of tyrosyl phosphorylation of PAK1 in breast cancer cell growth. We have demonstrated that tyrosyl phosphorylation of PAK1 is essential for maximal E2-dependent breast cancer cell proliferation. We were able to conclude this as a result of our proliferation experiment in which overexpression of phospho-tyrosyl-deficient PAK1 Y3F in MCF7 and T47D cells inhibited E2-dependent cell proliferation (Figure 25). The activity of PAK1 was found to be an important factor in E2-induced cell proliferation because IPA3, which is a PAK1 inhibitor, inhibited E2-induced breast cancer cell proliferation. In order to begin to elucidate the difference in E2 response between cells overexpressing WT PAK1 and PAK1 Y3F, we assessed E2-dependent PAK1 activation. PAK1 kinase activity, as assessed by *in vitro* kinase assay, showed that WT PAK1 activity increased in response to PRL in a time-dependent manner. PAK1 Y3F activity was decreased in response to PRL and this effect has been
previously reported (Hammer et al., 2015b; Rider et al., 2013b). These data suggest that PRL increased PAK1 kinase activity and tyrosines 153, 201 and 285 are responsible for this activation. In agreement with previously published data, we observed an increase in WT PAK1 kinase activity in response to E2. Interestingly, mutation of tyrosines 153, 201 and 285 led to complete inhibition of E2-stimulated PAK1 activity. In support of this, assessment of Thr423 on PAK1 (autophosphorylation site that indicates PAK1 activation) in response to E2 confirmed that E2 stimulates WT PAK1, but not PAK1 Y3F activation. This suggests that tyrosyl phosphorylation of PAK1 is important for E2-stimulated PAK1 activation. Both WT PAK1 and PAK1 Y3F were activated similarly by HRG indicating that PAK1 Y3F remains functionally active; however, tyrosyl phosphorylation-dependent activation is altered.

To begin to uncover the tyrosine kinase responsible for E2-dependent PAK1 tyrosyl phosphorylation, we examined JAK2. Tyrosines 153, 201 and 285 were identified as JAK2 phosphorylation sites on PAK1; therefore, it is logical to expect JAK2 to be activated by E2 and mediate PAK1 tyrosyl phosphorylation. Surprisingly, JAK2 was not activated by E2, but PAK1 is tyrosyl phosphorylated indicating that other tyrosine kinase(s) are responsible for PAK1 tyrosyl phosphorylation.

There has been previous evidence supporting the role of various tyrosine kinases regulating for PAK1 in various contexts. For example, the cytoplasmic tyrosine kinase Etk/Bmx of the Tec family of non-receptor tyrosine kinases associates with and phosphorylates PAK1, subsequently activating PAK1 (Bagheri-Yarmand, Mandal et al. 2001). Additionally, in constitutively activated v-Erb receptor-transformed cells, a very active and tyrosyl-phosphorylated form of PAK1 was found (McManus et al., 2000).
Another PAK family member, PAK2, is regulated by Src-dependent tyrosyl phosphorylation at tyrosine 130. In contrast, another non-receptor tyrosine kinase, c-Abl, phosphorylates PAK2, but causes a decrease in PAK2 kinase activity (Renkema et al., 2002). We hypothesized that one or more of the above mentioned tyrosine kinases (Etk, Erb, Src and c-Abl) would mediate PAK1 tyrosyl phosphorylation in response to E2. We found that Etk mediates PAK1 tyrosyl phosphorylation in response to E2. This conclusion is supported by several observations: 1) inhibition of Etk kinase by inhibitor AG897 was accompanied by concurrent inhibition of E2-dependent PAK1 tyrosyl phosphorylation and PAK1 kinase activity; 2) depletion of endogenous Etk by Etk specific siRNA resulted in complete inhibition of E2-dependent PAK1 activity; 3) reexpression of WT Etk reconstitutes E2-dependent PAK1 activation, while reexpression of kinase-dead Etk failed to recover E2-dependent PAK1 activity. Etk is activated by physiologically relevant growth factor in breast cancer cells and stimulate proliferation, anchorage-independent growth and tumorigenicity of breast cancer cells (Bagheri-Yarmand et al., 2001). Etk together with PAK1 play an important role in the regulation of mammary epithelial cancer cells.

Our finding that E2-stimulated PAK1 kinase activity is following Etk kinase activation is important, as it implies that Etk kinase constitutes an initial signal for PAK1 activation. PAK1 tyrosyl phosphorylation has been implicated in regulation of the actin cytoskeleton dynamic and cell motility, Rac-dependent and –independent PAK1 activation, enhancing PAK1 protein-protein interaction, and in the present study, implicated in the regulation of breast cancer cell proliferation. All these together underscore the significance of tyrosyl phosphorylation of PAK1 in cellular functions. Our
findings taken together with previously published data suggest that tyrosyl phosphorylation of PAK1 plays an important role in regulating PAK1 activity.

The rapid activation of PAK1 by E2 (Fig.12) indicates involvement of either membrane-localized estrogen receptor (mER) and/or GPER1. These two estrogen-responsive receptors are structurally different and can trigger different signaling pathways in the same cell including MCF-7 cells (Ariazi et al., 2010; Carmeci et al., 1997; Filardo et al., 2000). In order to confirm this, the activation of PAK1 was compared in MCF7 cells and TMX2-28 cells (ER and GPER1 negative clones of MCF7). PAK1 activation in response to E2 was only observed in MCF7 cells. Both cell lines, which are PRL receptor positive, responded similarly to PRL. In order to distinguish which of the E2-responsive receptors mediates E2-dependent PAK1 activation, SKBR3 cell line was used. SKBR3, which are ERα negative, but GPER1 positive, responded to E2 stimulation and PAK1 activation similar to that in MCF7 cells was observed suggesting that the E2 utilizes GPER1 to mediate its rapid, non-classical actions. To further delineate this signaling cascade, ERα and GPER1 were knocked-down either individually or together in MCF7 cells. Knock-down of either ERα or GPER1 decreased E2-dependent PAK1 activity by 50%. Interestingly, Double knock-down of both ERα and GPER1 in MCF7 cells completely abolished E2-dependent activation of PAK1 to basal level, but these cells still responded to HRG. These data highlight the versatility of E2. In SKBR3 cells that no longer express ERα, the rapid signaling of E2 have shifted and are being mediated by GPER1; in MCF7 cell, however, where both ERα and GPER1 are being expressed, E2 splits its signaling action to both receptors. These data uncover another important idea that in breast cancers that are ER negative, the absence of ERα
does not necessarily mean that E2 is not contributing to the progression of the disease. Taking into account that one in four patients with ER-positive tumors does not respond to anti-estrogens (Wittliff, 1984), the existence of an alternative estrogen receptor as GPER1, may provide the basis for a better understanding of mechanisms by which estrogens/anti-estrogens stimulate the proliferation of hormone-sensitive cancer cells, including breast carcinoma. Further supporting these findings, the expression of GPER1 has been found associated with the development of tamoxifen resistance in breast cancer patients (Ignatov et al., 2010; Mo et al., 2013). The activation of PAK1, among other intracellular signals, contributes to breast cancer cell proliferation. It is, therefore, important to include GPER1 in the profile of receptor being tested during determination breast cancer subtype for a more effective treatment option.

How do the membrane localized estrogen-responsive receptors transduce their signals? Membrane-localized ERα and GPER1 activate heterotrimeric G proteins (Nilsson et al., 2011). In an inactive state, the heterotrimeric G proteins (Goβγ) are in complex and associated with GPCR. Once activated, the G proteins dissociate from the receptor and one another to form a Ga and Gβγ dimer. Gβγ dimer directly binds to both PAK1 (Leeuw et al., 1998) and E2-activated mERα (Kumar et al., 2007). PAK1 can be activated by β1γ2 or β1γ5 subunits via the PI3-kinase and Akt pathways independently of Rac1 or Cdc42 (Menard and Mattingly, 2004). In that study EGF, PMA, LPA and carbachol were unable to augment PAK1 activity beyond that induced by β1γ2 or β1γ5 (Menard and Mattingly, 2004). Our data demonstrated additive an effect of E2 underlying a high degree of specificity for this pathway. The number of heterotrimeric G protein subunits variants identified includes 27 Ga, 5 Gβ, and 14 Gγ subunits giving a theoretical
1890 possible combination. G protein specificity have been described, and first level of specificity is dictated by the Ga subunit, which determines G protein effector specificity. Another possibility is that it is the heterotrimer that dictates specificity, not solely one subunit. There is much greater sequence divergence between members of Gβ and Gγ families (30–80% amino acid identity) than for the members of the Ga family (over 80% amino acid identity). The diversity of Gβγ subunits could explain how receptors might favor certain G protein combinations over others (Albert and Robillard, 2002). Our results indicate that a higher order of Gβγ–effector specificity is present in breast cancer cells, and β1γ2 or β1γ5 preferentially augment of PAK1 activity in response to E2.

PAK1 has been found in adhesion complexes, and localization of PAK1 to adhesion sites is mediated by direct binding of a proline-rich motif to SH3 domain of PIX proteins (Delorme-Walker et al., 2011; Manser et al., 1997; Stofega et al., 2004). We have previously implicated PRL-dependent tyrosyl phosphorylation of PAK1 in the interaction of PAK1 with βPIX (Hammer et al., 2015b). In this study, we demonstrated that E2-dependent tyrosyl phosphorylation of PAK1 increases βPIX-PAK1 interaction because there was no increased interaction between βPIX and PAK1 Y3F in response to E2. βPIX have been shown to enhance PAK1 activity by recruiting PAK1 to close proximity of Cdc42 and Rac1. We showed that overexpression of βPIX enhanced E2-dependent activation of WT PAK1. Overexpression of βPIX did not alter PAK1 Y3F activity suggesting that the regulation of PAK1 activity by βPIX requires tyrosyl phosphorylation of PAK1.

In order to confirm the significance of βPIX in E2-dependent activation of PAK1, βPIX was depleted in MCF7 cells and PAK1 activity was assessed in response to E2. In
control cell treated with E2, PAK1 activity was significantly enhanced; however, PAK1 activity was abolished in cells with βPIX knockdown treated with E2. When WT βPIX was reexpressed in the βPIX knockdown cell, E2-dependent activation of PAK1 was reconstituted. This result underscores this critical role of βPIX in E2-dependent activation of PAK1. To further elucidate this pathway, GEF deficient and PAK1 binding deficient mutants of βPIX were reexpressed in βPIX knockdown cell. The GEF deficient mutant partially rescues PAK1 activation in response to E2, but the PAK1 binding mutant was unable to rectify PAK1 activation. These data suggest that βPIX binding to PAK1 is required for its activation and that GEF activity of βPIX is essential for maximum E2-dependent PAK1 activation. Furthermore, phosphorylation of three tyrosines on PAK1 is also required for βPIX binding in response to E2.

We have demonstrated that tyrosines 153, 201 and 285 play a role in activation of PAK1 by E2, ability of PAK1 to activate PKA, phosphorylate S305-ERα and, finally, enhance cell proliferation in response to PRL+E2. It has been proposed by Kim and colleagues that tyrosyl phosphorylation of these sites may disrupt the inhibitory conformation of PAK1 and/or maintain PAK1 active conformation because Tyr153 resides nearby Ser144 and Ser149, Tyr201 locates near Ser199 and Ser204 (all are authophosphorylation sites) and Tyr285, which lies in the kinase domain, may stabilize the interaction between K299 and ATP molecules (Kim et al., 2014). Although it should be confirmed by further structural studies, this proposition is very attractive. Another possibility is that pTyr-PAK1 may create additional docking sites to recruit SH2 domain-containing proteins to facilitate PAK1 functions. Either way, these tyrosine phosphorylation events are critical for PAK1 functions.
Another notable finding in this study is that Ser305-ERα is phosphorylated by PAK1 in response of PRL in the absence of estrogen. Phosphorylation of Ser305-ERα was previously implicated in tamoxifen resistance and a correlation between high level of PAK1 and tamoxifen sensitivity in breast cancer patients was described (Holm et al., 2009b; Rayala et al., 2006b). Our data predict that patients with augmented levels of PRL will likely develop tamoxifen resistance due to Ser305-ERα phosphorylation by PRL-activated PAK1. Furthermore, the synergistic effects of estrogen and PRL signaling described here is physiologically and therapeutically relevant since breast cells are constantly exposed to both hormones, but anti-estrogen therapy targets only the estrogen component.

Based on our findings and previous studies, we propose a model for PAK1 in estrogen- and PRL-triggered intracellular signaling leading to a synergistic effect of PRL and E2 on Ser305-ERα phosphorylation and cell proliferation (Figure 26). E2 activates membrane-localized ERα and GPER1 leading to activation of heterotrimeric G protein. Activated G protein dissociates into α and βγ subunits which both activate several effectors. Gαs stimulates AC, cAMP and subsequently activates PKA, which phosphorylates many substrates, including Ser305-ERα. Released β1γ2 and β1γ5 dimers of G protein bind to PAK1 which is tyrosyl phosphorylated by Etk. pTyr-PAK1 recruits and binds to βPIX, association with which further activates PAK1 in a positive feedback. Although PKA is the main kinase which phosphorylates Ser305-ERα in response to E2, PKA activity is reciprocally regulated by activated pTyr-PAK1. If cells are exposed to both PRL and E2, Ser305-ERα is phosphorylated by both PKA and pTyr-PAK1 resulting
in maximal signal. Furthermore, activation of S305-ER\textsubscript{α} leads to enhanced Ser118-ER\textsubscript{α} phosphorylation, presumably due to an intramolecular conformational change of ER\textsubscript{α} (Michalides et al., 2004; Rayala et al., 2006b), and finally induces cell proliferation.

Tyrosyl phosphorylation of PAK1 by Etk in response to E2 and by JAK2 in response to
PRL is important for this cascade because Y3F mutant fails to establish this self-amplifying cascade and promote cell proliferation. We introduce PAK1 as a common node for estrogen- and PRL-dependent pathways making it an attractive target for anti-cancer therapy.
B. Derivative of differentiation-inducing factor-3 inhibits PAK1 activity and breast cancer cell proliferation

Differentiation-inducing factors 1-3 (DIFs 1-3) are chlorinated bioactive compounds identified in the slime mold *Dictyostelium discoideum*, which induce stalk cell differentiation (Kay et al., 1989; Morris et al., 1987; Town et al., 1976). Among them, DIF-1 is the most active in inducing stalk-cell differentiation, while DIF-2 has less activity than DIF-1. DIF-3, which is the first metabolite formed during DIF-1 degradation, has the lowest activity to induce stalk cells differentiation (reviewed in (Kay et al., 1989; Kay et al., 1999); (Gokan et al., 2005)).

Although DIFs are differentiation factors, they are considered anti-tumor agents because they inhibit proliferation of mammalian tumor cells in vitro, including leukemia (Akaishi et al., 2004; Gokan et al., 2005; Kubohara, 1997; Kubohara, 1999; Shimizu et al., 2004), cervical (Takahashi-Yanaga et al., 2003), gastric (Kanai et al., 2003), and colon (Kubokura et al., 2015) human tumor cells. Activity of different DIFs to inhibit proliferation of mammalian tumor cells is converse to the activity demonstrated for differentiation of *D. discoideum* stalk cells; the most potent anti-tumor agent is DIF-3 (Gokan et al., 2005; Kubohara, 1999; Takahashi-Yanaga et al., 2003). Moreover, DIF-1 and DIF-3 inhibit tumor growth in mice in vivo (Kubokura et al., 2015; Takahashi-Yanaga et al., 2014).

As for the mechanism(s) underlying the actions of DIFs in tumor cells, different intracellular pathways have been implicated in anti-proliferative effect of DIFs. Multiple studies demonstrated that DIF-3 reduces the expression level of cyclin D1 mRNA by inhibiting Wnt/β-catenin signaling pathway (Kubokura et al., 2015) and also that DIF-1
and DIF-3 reduce the protein level of cyclin D1 by accelerating its degradation via activation of GSK-3β (Kubokura et al., 2015; Takahashi-Yanaga et al., 2003; Takahashi-Yanaga et al., 2014). In addition, DIF-1 inhibits proliferation of leukemia cells through inhibition of ERK signaling (Akaishi et al., 2004) and inhibition of STAT3 via MEK-ERK-dependent phosphorylation of Ser727-STAT3 (Kanai et al., 2003). It has also been shown that DIFs are direct inhibitors for PDE1 (calmodulin-dependent phosphodiesterase) (Shimizu et al., 2004). Taken together, these findings shed a light on possible mechanisms of anti-proliferative effect of DIFs on tumor cells. However, the precise molecular machinery underlying the anti-proliferative action of DIFs remains to be elucidated.

The serine/threonine kinase PAK1 which regulates different intracellular functions including proliferation, cytoskeletal dynamics, cell survival, and gene transcription (Molli et al., 2009) is altered either in expression and/or activation in various cancers, including brain, pancreas, colon, bladder, ovarian, hepatocellular, urinary tract, renal cell carcinoma, thyroid, and breast cancers (Eswaran et al., 2012; Kumar et al., 2006). Of these cancers, the role for PAK1 in breast cancer has been studied to the most extent (Kumar et al., 2006; Molli et al., 2009). Because of the role of PAK1 in the mammary oncogenic network, PAK1 has become one of the focal points of investigation into the mechanism and onset of human breast cancer, and PAK1 inhibition represents a plausible drug target in breast cancer treatment. Therefore, in this study we sought to further corroborate the potential of DIFs as anti-proliferative agents in breast cancer cells and identify a possible target of DIFs’ antiproliferative action.
We have previously demonstrated that PRL-activated JAK2 tyrosine kinase phosphorylates and activates PAK1, resulting in the activation of cyclin D1 promoter activity and invasion of human breast cancer cells (Rider et al., 2013b; Rider et al., 2007; Tao et al., 2011). We thus assessed an effect of DIF-3 on PAK1 kinase activity because DIF-3 is the most potent anti-tumor agent among the original DIFs (Gokan et al., 2005; Kubohara, 1999; Takahashi-Yanaga et al., 2003). We treated MCF-7 cells stably expressing HA-PAK1 with DIF-3 over a time course followed by PRL treatment for 20 min. Immunoprecipitated PAK1 was assessed for PAK1 kinase activity by an in vitro kinase assay with histone H4 as a substrate. As expected, PRL increased PAK1 kinase activity by 2.6-fold, which was inhibited by DIF-3 in a time-dependent manner and reached the basal level by 2 hrs (Figure 27A).

**Figure 27. DIF-3 and its derivatives inhibit PAK1 kinase activity in cells.** (A) MCF-7 cells overexpressing HA-tagged PAK1 were serum-deprived, pre-treated with or without 20 µM DIF-3 over a time course and stimulated with or without PRL (200 ng/ml, 20 min) to activate PAK1. HA-PAK1 was immunoprecipitated and assessed for kinase activity as in Figure 12. (B) MCF-7 PAK1 cells were serum-deprived, pre-treated for 2 hrs with vehicle or 20 µM DIFs and their derivatives as indicated, followed by PRL treatment (200 ng/ml, 20 min). HA-PAK1 was immunoprecipitated with anti-HA Ab, subjected to an in vitro kinase assay.
Next, we compared the inhibitory effect of some other DIF derivatives on PRL-dependent PAK1 kinase activity. The cells were incubated with the DIF derivatives (20 µM) for 2 hrs prior to PRL treatment. PAK1 kinase activity was inhibited by all the DIF derivatives to different degree with exception of DIF-2 and THPH (Figure 27B).

We decided to study whether the effect of DIF-3(+1), which has the strongest inhibitory effect of PAK1 activity, depends on a ligand. As demonstrated in Figure 28A, PAK1 kinase activity was inhibited by DIF-3(+1) to the basal level in response to all

![Figure 28. DIF-3(+1) inactivates purified PAK1 in vitro.](image-url)

(A) MCF-7 PAK1 cells were serum-deprived, pre-treated with vehicle or 20 µM DIF-3 (+1) for 2 hrs, and stimulated with or without PRL (200 ng/ml, 20 min), E2 (1 nM, 30 min) or HRG (30 ng/ml, 15 min). Immunoprecipitated HA-PAK1 was subjected to the in vitro kinase assay. (B) Purified GST-tagged PAK1 was subjected to in vitro kinase assay in the presence of DIF-3(+1) or THPH. The same membrane was probed with anti-PAK1 antibody for detection of GST-PAK1. Relative PAK1 kinase activity was then normalized by the amount PAK1 for each lane. The numbers at the bottom give the relative fold increase of PAK1 activity.
three PRL, E2 and HRG (Figure 28A). These data suggests that DIF-3(+1) may act directly on PAK1 protein regardless of upstream effector.

To determine whether DIF-3(+1) directly inhibits PAK1, exogenous purified GST-PAK1 was combined with DIF-3(+1) or with biologically inactive THPH and the level of PAK1 autophosphorylation was assessed by the in vitro kinase assay as described above but in the absence of H4 substrate. Both concentrations of DIF-3(+1), 5 µM and 10 µM, inhibited PAK1 autophosphorylation to the same degree while THPH had no effect (Figure 28B). These data suggest that DIF-3(+1) directly inhibits PAK1 in vitro.

We have previously shown that, in response to PRL, tyrosyl phosphorylated PAK1 activates cyclin D1 promoter activity (Tao et al., 2011). Therefore, we sought to determine whether DIF-3(+1)-dependent inhibition of PAK1 kinase activity plays a role in cyclin D1 regulation in response to PRL, E2, EGF and HRG. First, we measured the induction of cyclin D1 promoter activity in MCF-7 PAK1 cells treated with or without PRL, E2, EGF and HRG. As shown in Figure 29, the cells transfected with cyclin D1 promoter-luciferase construct demonstrated increased luciferase expression in response to PRL, E2, EGF and HRG as expected, with the greatest effect of HRG. However, when
cells were treated with DIF-3(+1), induction of cyclin D1 promoter activity was significantly decreased for all four ligands studied (Figure 29). These data suggest that DIF-3(+1) negatively regulates the activity of the cyclin D1 promoter.

First, to confirm the role of PAK1 in cell proliferation, we treated MCF-7 PAK1 and T47D PAK1 cells with cell-permeable small-molecule PAK1 inhibitor IPA-3.

![Graph showing cell proliferation](image)

**Figure 30. DIF-3(+1) inhibits breast cancer cell proliferation.** (A) MCF-7 and T47D cells overexpressing PAK1 were serum-deprived and incubated with vehicle (vcl), PRL (500 ng/ml) in presence or absence of IPA-3 (5µM). MCF-7 (B) or T47D (C) cells overexpressing PAK1 were serum-deprived and incubated with vehicle or 5 µM DIF-3(+1) in the presence of either vehicle, 500 ng/ml PRL, 1 nM E2, 10 ng/ml EGF or 30 ng/ml HRG. Cell density was assessed after seven days by MTT cell proliferation assay. Changes in cell numbers at day 7 are shown as percentages of the vehicle-treated cell number. Bars represent mean ±S.E., *, p <0.05 compare with cells in the same condition treated with vehicle, n=3.
IPA-3 inhibited PRL-dependent proliferation of both MCF-7 and T47D cells (Figure 30A). Next, as shown in Figure 30B and C, PRL, E2, EGF and HRG induced proliferation of both MCF-7 and T47D cells to different degrees which probably depends on the amount of receptors to be activated by a ligand. We have shown that DIF-3(+1) strongly inhibited ligand-induced proliferation of both MCF-7 and T47D cells (Figure 30B and C). Both cell lines were assessed for cytotoxicity for DIF-3(+1), and the concentration used was not toxic (data not shown).

**Discussion**

THPH is a non-bioactive analog of DIF-1 which affects neither cell growth nor cell differentiation (Akaishi et al., 2004; Gokan et al., 2005; Kubohara, 1997; Kubohara, 1999). Both DIF-1 and DIF-3 exhibit anti-proliferative activities in different human cancer cells, as was described in the Introduction section, and indeed both DIF-1 and -3 inhibit PAK1 activity. Meanwhile, DIF-2, which does not inhibit proliferation in human leukemia K562 cells (Gokan et al., 2005), also does not inhibit PAK1 in our assay. We have demonstrated that DIF-3 inhibits PAK1 stronger than DIF-1 (63% inhibition by DIF-3 vs. 44% by DIF-1) (Figure 27B). DIF-3 has been shown to exert a more powerful anti-proliferative effect than DIF-1 in human cervical cancer cells (Takahashi-Yanaga et al., 2003), leukemia (Akaishi et al., 2004; Kubohara, 1997), and colon cancer cells (Kubokura et al., 2015). Interestingly, the order of inhibitory effect of DIFs on PAK1 in MCF-7 cells described herein corresponds to the order of their potency to inhibit proliferation of human leukemia K563 cells: DIF-3 > DIF-1 > DIF-2 and THPH (Kubohara, 1999). Since DIF-2 was ineffective in PAK1 kinase assay, hexanone (the alkyl group in DIF-1) rather than pentanone in DIF-2 (Kubohara, 1999) appeared to be
important for inhibition of PAK1. Because chemically modified derivatives of DIF-3 (Bu-DIF-3) has been shown to be a more potent anti-proliferative agent than DIF-3 (Gokan et al., 2005), we tested the effect of different derivatives of DIF-1 and DIF-3 on PAK1 activity and demonstrated that DIF-3 derivatives DIF-3(3M) and DIF-3(+1) inhibit PAK1 kinase activity to the maximal degree in MCF-7 cells (78% inhibition) (Figure 27B).

PAK1 is activated in response to PRL, estrogen and heregulin (a ligand for HER3 (human epidermal growth factor receptor-3) and HER4 (human epidermal growth factor receptor-4)) by different signaling pathways (Adam et al., 1998; Mazumdar and Kumar, 2003b; Rider et al., 2007). PAK1 has been previously implicated in induction of cyclin D1 expression (Balasenthil et al., 2004a; Balasenthil et al., 2004b). Balasenthil et. al proposed that active PAK1 can increase cyclin D1 transcription through two independent pathways – the NFκB pathway and phosphorylation of S305 of estrogen receptor alpha (Balasenthil et al., 2004a; Balasenthil et al., 2004b). We have previously demonstrated that, in response to PRL, tyrosyl phosphorylated PAK1 translocates into the nucleus to activate cyclin D1 promoter activity and that adapter protein Nck keeps PAK1 into the cytoplasm, preventing activation of cyclin D1 promoter activity (Tao et al., 2011). As DIF-3 induces nuclear translocation of GSK-3β (Takahashi-Yanaga et al., 2003), we may speculate that DIF-3(+1) affects intracellular localization of PAK1 to retain PAK1 in the cytoplasm leading to inhibition of cyclin D1 expression.

Many factors, including PRL, E2, EGF, HRG, as well as activated PAK1 induce cyclin D1 expression ((Balasenthil et al., 2004a; Brockman et al., 2002); reviewed in (Fu et al., 2004)) while DIF-1 and DIF-3 decrease cyclin D1 expression level by inducing
cyclin D1 proteolysis via activation of GSK-3β (Kubokura et al., 2015; Takahashi-Yanaga et al., 2003; Takahashi-Yanaga et al., 2014).

DIFs exhibit powerful anti-proliferative effect in various cancer cells, including leukemia (Akaishi et al., 2004; Gokan et al., 2005; Kubohara, 1997; Kubohara, 1999; Shimizu et al., 2004), cervical (Takahashi-Yanaga et al., 2003), gastric (Kanai et al., 2003), and colon (Kubokura et al., 2015) cells. However, the effect of DIFs on breast cancer cells has not been studied. In this study, we examined whether DIF-3(+1) inhibits the proliferation of breast cancer cells.

Based on our data presented here and previous studies, we suggest that DIF-1 and -3 inhibit PAK1 kinase in cells. We have found that DIF-3(+1) derivative inhibits PAK1 kinase activity both in cells and in vitro that leads to negative regulation of cyclin D1 promoter activity and inhibition of cell proliferation. We propose PAK1 as a DIF-3(+1) target which mediates the anti-proliferative effect of DIF-3(+1) on breast cancer cells.
C. PAK1 regulates breast cancer cell invasion through secretion of matrix metalloproteinases in response to prolactin and three-dimensional Collagen IV

Breast cancer is one of the most common malignancies affecting women. Colonization of distant tissues by tumor cells represents the most dangerous attribute of cancer. One hallmark of invasive breast cancer cells is increased expression and activity of matrix metalloproteinases (MMPs) that contribute to invasive potential. MMPs are a family of Zn$^{2+}$-dependent enzymes composing 23 members. MMP-1 (Collagenase 1) specifically degrades Collagen I, a major component of the extracellular matrix (ECM) and other fibrillar Collagens. MMP-1 is critical for the ECM remodeling. In clinical studies, increased MMP-1 expression is associated with advanced stages of breast cancer and may be a predictive marker for invasive disease (Poola et al., 2005). MMP-3, or stromelysin 1, degrades a variety of ECM substrates, including Collagens. MMP-3 is up-regulated in breast tumors and contributes to cancer development. Indeed, mice overexpressing MMP-3 show excessive side-branching and eventual formation of mammary tumors (Sternlicht et al., 2000). MMP-2 and MMP-9 are both type IV Collagenases that contribute to tumor invasion in vitro because of their ability to break down basement membrane, degrading Collagen IV in particular. Elevated circulating MMP-9 levels are apparent in breast cancers and MMP-2 and/or MMP-9 release is associated with tumor invasion and metastasis (Lu et al., 2011; Overall and Lopez-Otin, 2002). The expression of MMPs is regulated at the transcriptional and posttranscriptional levels (including the stability of mRNA and protein, as well as the release and activation of protein) by hormones, growth factors, and cytokines. Despite efforts to discover the
cellular pathways regulating MMPs, little is known as to how different cytokines cooperate with cytoskeletal proteins to regulate MMPs expression.

Cells adhere to the ECM throughout most of their lifetime. The molecular composition of the ECM, specific association of multiple growth factors/cytokines with the matrix, and “dimensionality” play major roles in the response of cells to their local matrix microenvironment (Geiger and Yamada, 2011). Three-dimensional (3D) matrix is a critical component of mammary tissue development not only under physiologic but also in pathophysiologic conditions. In vivo, women with dense mammary tissue, associated with an increasing amount of Collagen in the stroma, are at 4–6 times greater risk of breast cancer and have a poor prognosis (Boyd et al., 1998; Colpaert et al., 2001). In vitro, increasing 3D matrix tension affects mammary cell morphogenesis and physiologic functions. Furthermore, reciprocal interactions between mammary epithelial cells, ECM, and ECM-remodeling enzymes are critical for development and differentiation during mammary gland development. Loss of this interaction leads to tumor progression (Adriance et al., 2005).

Prolactin (PRL), a hormone of the GH/cytokine family, exerts both the endocrine and autocrine/paracrine effects and functions in both reproduction and as a cytokine exerting profound effects on a wide range of tissues, with more than 300 effects described in vertebrates (Ben-Jonathan et al., 2008; Bernichtein et al., 2010). PRL binding to its receptor activates tyrosine kinase JAK2, PRL receptor phosphorylation, and the phosphorylation of signal transducer and activator of transcription (STAT) 5a and 5b, STAT3, and STAT1. PRL also activates MAPKs, including ERK1/2, ERK5, p38 and c-Jun N-terminal kinase (JNK) 1/2, protein kinase C, and PI-3 kinase (Carver et al., 2009).
There is now clear evidence that PRL is involved in breast cancer (Clevenger, 2003; Tworoger and Hankinson, 2006). PRL increases motility of breast cancer cells (Maus et al., 1999). These data, combined with animal studies reporting either increased metastases with PRL administration (Liby et al., 2003) or prevention of neoplasia progression into invasive carcinoma in PRL receptor deficient mice (Oakes et al., 2007), suggest that PRL is involved in the development of metastasis and tumor progression although the exact mechanism remains to be clarified. In contrast, PRL was reported to act as a suppressor of breast cancer cell invasion in vitro (Nouhi et al., 2006; Sultan et al., 2005), indicating that the role of PRL in breast cancer invasion is yet to be clarified thus, must be explored further.

As have been discussed earlier, PAK1 is a major component of breast cancer progression (Balasenthil et al., 2004b; Li et al., 2008; Mira et al., 2000; Salh et al., 2002). Activated PAK1 increases invasion of breast cancer cells, and expression of a kinase-dead PAK1 mutant in highly invasive breast cancer lines leads to reduced invasiveness (Adam et al., 2000). Conversely, hyperactivation of the PAK1 pathway in the noninvasive breast cancer cell line MCF-7 promotes cell migration and anchorage-independent growth (Vadlamudi et al., 2000). We have shown that PAK1 is a JAK2 substrate and that PRL-activated JAK2 phosphorylates PAK1 in vivo on tyrosines 153, 201, and 285 (Rider et al., 2007). It has recently been demonstrated that the PAK1 substrate filamin A (FLNa) plays a role in PRL-dependent cell motility (Hammer et al., 2013; Rider and Diakonova, 2011). Therefore, we aimed to understand the role of tyrosyl phosphorylated PAK1 in breast cancer cell invasion.
It has been previously demonstrated that the JAK2 phosphorylation of PAK1 tyrosines 153, 201, and 285 promotes PRL-dependent changes in the actin cytoskeleton and cell motility (Rider and Diakonova, 2011; Rider et al., 2007). To assess the significance of tyrosyl-phosphorylated PAK1 (pTyr-PAK1) in breast cancer cell invasion, we used TMX2–28 cells, a variant of the MCF-7 cell line (Fasco et al., 2003). These cells are PRL responsive and highly invasive compared with poorly invasive MCF-7 cells (Gozgit et al., 2006). We have established TMX2–28 cell lines that stably express green fluorescent protein (GFP) alone (as vector control) or with either myc tagged PAK1 WT or a PAK1 Y3F mutant in which the 3 JAK2 phosphorylation sites (tyrosines 153, 201, and 285) were mutated to phenylalanine.

To determine whether PAK1 Y3F protein was functional and activated by heregulin, myc-tagged PAK1 WT and PAK1 Y3F were immunoprecipitated (IP’d) from cell lines treated with HRG (30 ng/ml, 15 min) and their kinase activities were measured in an in vitro kinase assay with H4 histone as a substrate. Heregulin (HRG), a ligand for HER3 (human epidermal growth factor receptor-3) and HER4 (human epidermal growth factor receptor-4), activates PAK1 in MCF-7 cells (Adam et al., 1998). As shown in Figure 31A, HRG-induced PAK1 kinase activity and autophosphorylation were similar in the cells expressing PAK1 WT and PAK1 Y3F.

To determine whether PRL treatment altered PAK1 kinase activity, TMX2–28 clones were treated without or with PRL, and PAK1 kinase activity was measured using an in vitro kinase assay. PRL treatment more than doubled PAK1 WT kinase activity, compared with untreated control (Figure 31B). PRL can activate both PAK1WT and PAK1 Y3F through Rac1 (PRL activates Rac1 (Miller et al., 2005)). However, in the
presence of PRL, the kinase activity of PAK1 WT was significantly stronger than PAK1 Y3F (Figure 31B). These data demonstrated that PAK1 WT and PAK1 Y3F have similar kinase activities in response to HRG. PRL stimulates both PAK1 WT and PAK1 Y3F probably through Rac1/Cd42 and further increases PAK1 WT kinase activity, suggesting that tyrosines 153, 201, and 285 are involved in regulating this enhanced activity.
Because PAK1 activation is closely linked with the invasiveness of MCF-7 cells (poorly invasive), as well as MDA-MB435 and MDA-MB231 cells (highly invasive) (Adam et al., 1998; Adam et al., 2000), we asked whether the PRL-dependent tyrosyl phosphorylation of PAK1 is also required for the maintenance of the invasive phenotype in breast cancer cells. The effect of pTyr-PAK1 WT on PRL-induced invasion was demonstrated by evaluating migration through Matrigel (Figure 32). Overexpression of

![Fig 32](image)

**Figure 32. Maximal invasion of TMX2–28 cells in response to PRL requires tyrosyl phosphorylation of PAK1.** A, TMX2–28 cells stably overexpressing GFP, PAK1 WT, or PAK1 Y3F were loaded into the upper part of the Boyden chamber coated with the Matrigel. The number of cells that migrated to the lower surface of the chamber toward PRL (100 ng/mL) (black bar) or buffer control (white bar) after 48 hours were counted in 5 random fields and plotted. B, TMX2–28 cells were transfected with control or PAK1 siRNA and assessed for invasion as in panel A. Silencing efficiency was judged by immunoblotting with anti-PAK1 Ab 48 and 72 hours after transfection. The expression levels of actin were used as an internal control. Bars represent mean ± SE. *, P < .05 compared with untreated cells. Each experiment was repeated three times. Scale bar, 300 µm. Experiments were performed by Oladimeji, P., Rider, L., Diakonova, M.
PAK1 WT accelerated migration in response to PRL (78 invaded cells) as compared with cells overexpressing GFP (51 invaded cells), whereas overexpression of PAK1 Y3F significantly inhibited cell invasion (35 invaded cells) (Figure 32A). These data suggest that PAK1 Y3F inhibits cell invasion and implicates tyrosyl phosphorylation in the potentiating effect of PAK1 on cell invasiveness. To directly establish the significance of PAK1 signaling on PRL-dependent cell invasion, we next examined the effect of knockdown of endogenous PAK1 by PAK1-specific small interfering RNA (siRNA). We found that PAK1-depleted cells maintained basal invasion (white bars in plots in Figure 32B) but demonstrated ablation of PRL-induced migration (black bars in plots in Figure 32B).

An important step in the invasion process is the destruction of ECM by MMPs. To test whether altered secretion of MMPs by PRL-activated PAK1 regulates the invasion of TMX2–28 cells, we assessed the secretion of MMP-1, MMP-2, MMP-3, and MMP-9, because these MMPs play an important role in breast cancer invasion and metastasis (Folgueras et al., 2004; Radisky and Radisky, 2010). Both Collagen I or IV induced MMP transcription and/or secretion, and therefore we plated cells onto dishes covered with either Collagen I or IV. Because a role of the 3D Collagen lattice in the transcriptional control and/or activation of different MMPs has been demonstrated previously (Ellerbroek et al., 1999; Haas et al., 1998; Zhou et al., 2009), we also embedded the cells in either Collagen I or IV, after which we treated them with or without PRL and measured MMP secretion (Figure 33). In TMX2–28 lines plated on plastic, Collagen I or IV, a moderate increase in MMP-1 release was observed in PAK1 WT cells treated with PRL, but plating cells on either type of Collagen had no effect.
Figure 33. Collagens and PRL-dependent tyrosyl phosphorylation of PAK1 regulate secretion of invasive-relevant MMPs. TMX2–28 cells stably overexpressing GFP, PAK1 WT, or PAK1 Y3F were plated on plastic, on plastic covered with Collagen I, embedded in Collagen I, on plastic covered with Collagen IV, or embedded in Collagen IV. After 24 hours of serum deprivation, the cells were treated with (black bars) or without (white bars) 100 ng/mL PRL for 48 hours, and the conditioned medium was assessed for protein level of secreted MMP-1 (A), MMP-3 (B), MMP-2 (C), and MMP-9 (D) by ELISA. Bars represent mean ± SE. *, P < .05 compared with the same cells untreated with PRL. #, P < .05 compared with cells expressing GFP with the same treatment. Each experiment was repeated 3 times. Experiments performed by Rider L.

(Figure 33A). In contrast, cells overexpressing PAK1 WT and embedded in Collagen IV exhibited dramatically induced MMP-1 secretion (white bar for WT cells embedded in
Collagen IV in Figure 33A); treatment of these cells with PRL further stimulated MMP-1 secretion (black bar). 3D Collagen IV did not affect MMP-1 secretion in the cells overexpressing GFP and PAK1 Y3F regardless of PRL treatment, suggesting that tyrosines 153, 201, and 285 of PAK1 are required for the effect of Collagen IV on MMP-1 secretion. Interestingly, cells embedded in Collagen I also exhibited enhanced MMP-1 secretion (Figure 33A), although not as strong as 3D Collagen IV (2-fold increase for WT cells treated with PRL as compared with GFP cells treated with PRL for 3D Collagen I vs 15-fold increase for the WT cells embedded in Collagen IV). Collectively, TMX2–28 cells overexpressing PAK1 WT, treated with PRL, and embedded in Collagen IV exhibited the strongest effect on MMP-1 release as compared with control GFP and PAK1 Y3F cells or PAK1 WT cells grown on the surface of Collagen IV. MMP-3 regulation by PRL-activated PAK1 and the different substrates was similar to that observed for MMP-1. 3D Collagen IV significantly increased MMP-3 secretion, especially when combined with PRL treatment (Figure 33B). This effect was again pTyr-PAK1 PRL-dependent dependent because PAK1 Y3F cells failed to induce MMP-3 secretion in response to 3D Collagen IV with or without PRL (Figure 33B).

We next assessed MMP-2 secretion in TMX2–28 cell clones plated on either plastic, Collagen I, Collagen IV, or embedded in Collagen I or IV. Embedding cells in 3D Collagen IV induced MMP-2 secretion relative to the other substrates (Figure 33C). Without PRL treatment, this effect was PAK1-independent (compare white bars for GFP, PAK1 WT, and PAK1 Y3F cells for each type of substrate). However, after PRL treatment, control GFP cells and cells overexpressing PAK1 WT embedded in Collagen IV secreted significantly less MMP-2 than without PRL (compare black and white bars.
for GFP and WT cells embedded in Collagen IV in Figure 33C). In contrast, PRL had no effect on MMP-2 secretion by PAK1 Y3F cells, confirming the dominant-negative role of this mutant in the regulation of PRL-PAK1-dependent MMP-2 secretion.

We assessed secretion of MMP-9 under the conditions described above. In contrast to MMPs 1, 2, and 3, secretion of MMP-9 was PRL-independent (compare white and black bars in Figure 33D) and negatively regulated by PAK1. WT PAK1 and PAK1 Y3F both inhibited MMP-9 secretion. Neither 2D nor 3D Collagen IV affected MMP-9 secretion, whereas 3D Collagen I dramatically induced secretion (3-fold increase in GFP cells embedded in Collagen I as compared with GFP cells plated on plastic), which is in contrast to MMPs 1, 2, and 3.

To assess the role of PAK1 in PRL-induced MMP secretion, we knocked down PAK1 in TMX2–28 cells and evaluated secretions of MMP-1, -2, -3, and -9. We found

![Graphs showing MMP-1, MMP-3, MMP-2, and MMP-9 secretion](Figure 34)

**Figure 34.** PAK1 is essential for secretion of invasive-relevant MMPs. TMX2–28 cells were transfected with control or PAK1 siRNA, stimulated (black bars) or not (white bars) with PRL, and the conditioned medium was assessed for secreted MMP-1 by ELISA. Bars represent mean ± SE. *, P < .05 compared with the same cells untreated with PRL. #, P < .05 compared with same cells treated with PRL. Each experiment was repeated 3 times. Experiments by Oladimeji P. and Diakonova M.
that PAK1-depleted cells exhibited decreased basal and PRL-induced secretion of MMP-1, -2, and -3 (Figure 34). Interestingly, secretion of MMP-9 was stimulated by the absence of PAK1, which is in agreement with our data on the inhibitory effect of PAK1 on MMP-9 production (Figure 33D).

Our data suggest that 3D Collagen IV strongly stimulates the secretion of MMP-1 and -3 via a PAK1-dependent pathway and that PRL treatment augments this effect via the PAK1 tyrosyl phosphorylation. 3D Collagen IV also stimulates MMP-2 secretion that was inhibited by pTyr-PAK1. In contrast, MMP-9 secretion is PRL-independent and stimulated by 3D Collagen I (but not IV), and inhibited by PAK1.

Data from the literature suggest that MMP transcription is regulated through multiple signaling pathways, including MAPK ERK1/2, p38 and JNK1/2, and NFκB signaling (Overall and Lopez-Otin, 2002). Because PAK1 can modulate each of these pathways (Molli et al., 2009), and because PRL also stimulates MMP transcription (this study), we sought to investigate the involvement of these signaling molecules in the PRL-PAK1 regulation of MMP secretion. To investigate the ability of PRL to activate MAPKs in TMX2–28 clones, the cells were treated over a time course with PRL, and cell lysates were analyzed for MAPK phosphorylation. PRL induced ERK1/2 phosphorylation faster in PAK1 WT and PAK1 Y3F clones as compared with the control cells, although the level of pERK1/2 was similar in all three clones by 30 minutes of PRL treatment (Figure 25A). The activation of JNK1/2 was apparent at 30 minutes and remained elevated for at least 140 minutes. Both PAK1 WT and PAK1 Y3F demonstrated significantly stronger activation of JNK1 and JNK2 as compared with the control GFP cells (Figure 35). Surprisingly, phosphorylation of p38 was strongly enhanced in PAK1 Y3F cells as
**Figure 35. PRL regulate multiple MAPKs.** TMX2–28 cells stably overexpressing GFP, PAK1 WT, or PAK1 Y3F were serum deprived for 72 hours and treated with 100 ng/mL PRL for the time indicated. Cells were lysed, and proteins were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. The graphs represent densiometric analysis of the band obtained for phosphorylated MAPKs normalized with total MAPKs for each lane. *, P < .05 compared with cells expressing GFP with the same treatment. Each experiment was repeated 3 times.
compared with the PAK1 WT and GFP cells (Figure 35). These data suggest that both PAK1 and PAK1 Y3F stimulate activation of JNK1/2 and enhanced early activation of ERK 1/2 in response to PRL. In contrast, phosphorylation of one or more of these 3 tyrosines on PAK1 inhibits p38 activation suggesting that PAK1-dependent regulation of PRL response is a combination of positive phospho-tyrosyl-independent effect of PAK1 on JNK1/2 and ERK1/2 activation and negative phospho-tyrosyl-dependent effect of PAK1 on p38 activation.

To investigate the role of PRL-activated MAPKs in cell invasion, we used selective MAPK inhibitors. Because PD98059 only moderately inhibited PRL-induced ERK1/2 phosphorylation (Gutzman et al., 2004b), we used U0126. U0126 (10 µM) blocked the PRL-induced ERK1/2 phosphorylation in 1 hour (Figure 36A). SB 203580

![A](image1)

**Figure 36** Selective Inhibitors Inhibit PRL-Activated Multiple MAPKs. (A) TMX2–28 cells were serum deprived for 72 hours and treated with 100 ng/mL PRL and the following inhibitors as indicated. Cells were lysed, and proteins were resolved by SDS-PAGE and analyzed by immunoblotting (B) TMX2–28 cells stably overexpressing GFP, PAK1 WT, or PAK1 Y3F were treated with TNF-α (10 ng/mL) for the time indicated and analyzed as in A. The bottom immunoblots: The TMX2–28 cells were preincubated with 5 µM inhibitor of IKK VII overnight and treated with TNF-α for the time indicated. The expression levels of γ-tubulin (α-tubulin) or actin were used as an internal control. Experiments performed by Oladimeji P. and Rider L.
(10 µM), a p38-selective inhibitor, blocked PRL-dependent activation of p38 in 6 hours whereas SP 600125 (30 µM), a JNK1/2-selective inhibitor, inhibited activation of JNK1/2 in 1 hour (Figure 36A). It has been shown that ERK5 plays a role in cell invasion (Mehta et al., 2003). Although UO 126 blocks EGF-stimulated phosphorylation of ERK5 in HeLa cells (Mody et al., 2001), it does not affect ERK5 phosphorylation in response to PRL (Gutzman et al., 2004b). In agreement with the latest, we have shown that PRL activated ERK5 over a time course (Figure 37A), and UO 126 (10 µM, 1 h) blocked the PRL-induced ERK1/2 phosphorylation, without affecting ERK5 phosphorylation (Figure 37B). We therefore used UO 126 to further investigate a role of PRL-dependent

**Figure 37. UO 126 inhibitor does not inhibit PRL-activated Erk5** (A) TMX2-28 cells were serum deprived for 72h and treated with 100 ng/ml PRL for 0, 5, 15, 30, 45, 135 min and 48h. Cells were lysed, proteins were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. (B) TMX2-28 cells were serum deprived for 72h, treated with UO 126 (10 μM, 1h) and PRL (100 ng/ml, 135 min). Cells were lysed, proteins were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.
activation of ERK1/2, but not ERK5, in the cell invasion. Because the MMP ELISAs required 48 hours of PRL treatment, we tested all inhibitors for 48 hours for impact on cell viability. SB203580 (10 µM) and U0126 (10 µM) did not affect viability over 48 hours whereas SP600125 (30 µM) was slightly toxic to the cells (data not shown). For ELISA, cells were preincubated with 30 µM SP600125 for 6 hours and then incubated with or without PRL for 48 hours in the presence of non-toxic concentration of 15 µM SP600125.

To demonstrate a mechanistic role of MMP-1, -2, -3, and -9 as well as MAPK pathways in PRL-induced cell invasion, we first knocked down MMP-1 in TMX2–28 clones and assessed the cell invasion through Matrigel. We found that all MMP-1-depleted cell lines maintained basal invasion (white bars in plots in Figure 38A) but were deficient in PRL-induced migration (black bars in plots in Figure 38A). Next, we inhibited MMP-2, MMP-3, and MMP-9 proteins with the selective MMP inhibitors. Because the invasion assay required 48 hours, we tested all inhibitors for 48 hours for impact on cell viability. MMP-3 inhibitor I (50 µM) and MMP-9 inhibitor I (50 µM) did not affect viability over 48 hours whereas MMP-2 inhibitor I (125 µM) was slightly toxic to the cells (data not shown). For invasion assay, cells were pre-incubated with MMP-2 (125 µM), MMP-3 (50 µM), or MMP-9 (50 µM) inhibitors overnight and then allowed them to invade with or without PRL for 48 hours in the presence of 72.5 µM MMP-2 inhibitor I, 50 µM MMP-3, or 50 µM MMP-9 (neither concentration was toxic). MMP-3 inhibitor I abolished invasion of PAK1 WT and control GFP cells whereas invasion of PAK1 Y3F cells was the same as without the inhibitor. Inhibition of MMP-2 led to decreased invasion of PAK1 WT cells and did not have an effect on invasion of PAK1
Y3F cells. MMP-9 inhibitor I had no effect on invasion of all 3 clones (Figure 38B).

These data suggest that MMP-1 is required for invasion of both PAK1 WT and PAK1 Y3F cells, whereas MMP-3 is required for, and MMP-2 contributes to, invasion of tyrosyl-phosphorylated PAK1 but not PAK1 Y3F cells.

To determine whether PRL-dependent PAK1 phosphorylation regulates cell invasion through ERK1/2, p38, or JNK1/2 pathways, we used selective MAPK inhibitors.

As shown in Figure 38C, PRL-induced cell invasion was abolished by ERK1/2 inhibitor
UO 125 in all 3 clones. Inhibition of JNK1/2 and p38 decreased invasion of GFP and PAK1 WT cells in response to PRL but not in PAK1 Y3F cells.

In summary, MMP-1 and MMP-3 proteins are required for PRL-regulated cell invasion whereas MMP-9 has no effect. ERK1/2, JNK1/2 and p38 are involved in PRL-regulated invasion to varying degrees.

Discussion

The data presented provide evidence that PAK1 stimulates the PRL-dependent invasion of TMX2–28 human breast tumor cells through Matrigel by up-regulating the expression and secretion of MMP-1, -2, and -3 in 3D Collagen IV, which makes up 31% of Matrigel protein composition. PRL-induced PAK1 tyrosyl phosphorylation leads to a further increase in MMP-1 and MMP-3 expression and modestly inhibits MMP-2 expression. The expression and secretion of MMP-9 are dramatically increased by 3D Collagen I and are not affected by PRL but inhibited by PAK1. MMP-1 and -3 are required for, and MMP-2 contributes to, PRL-dependent invasion. These data suggest that the induction of each MMP is complex and regulated differently by PRL-, PAK1-, and ECM-dependent pathways.

It is well documented that the interaction of cells with 2D substrates is significantly different than in more natural 3D environments that cells encounter in vivo (Nelson and Bissell, 2006). Cells embedded in 3D matrix have higher amounts of ligated matrix-receptors, compared with the cells grown on a thin film of matrix. Collagen receptors, such as integrins and DDRs (discoidin domain receptors), are signal transducing receptors. Integrin clustering initiates an array of signaling cascades, including activation of Rho family of small GTPases, MAPKs, and phosphatidylinositol
3-kinases that can regulate MMP expression (Daley et al., 2008). Collagen I induces expression/secretion of MMP-1 and MMP-9, whereas Collagen IV up-regulates MMP-2 and -9 expression (Castro-Sanchez et al., 2011; Cortes-Reynosa et al., 2008; Langholz et al., 1995; Zhou et al., 2009). Other Collagen receptors, DDR1 and DDR2, are tyrosine kinase receptors that bind to native Collagens and play integrin-independent roles in signaling, cell adhesion, matrix remodeling, and MMP secretion/activation (Vogel et al., 1997; Vogel et al., 2006). Thus, embedding cells in a 3D matrix can amplify signals from ligated integrins/DRRs and lead to MMP expression. Furthermore, “cross talk” and “synergy” between signaling by ECM receptors and growth factors and cytokines have been reported that involve cooperation in the downstream signal transduction pathways.

Another possible explanation of how 3D Collagen results in elevated expression of MMPs 1–3 and 9 as compared with 2D Collagen relates to the physical properties of 3D matrixes (Daley et al., 2008; Lu et al., 2011). The actin cytoskeleton appears to be the major cellular system for transduction of force generated by the external network. Cytoskeletal stretching correlates with the recruitment of adhesion complex proteins and triggers signals resulting in the induction of a matrix-degrading protease (Giannone and Sheetz, 2006). This may explain why 3D Collagen I induces expression of only MMP-9 whereas 3D Collagen IV upregulates expression of MMP-1, -2, and -3 but not MMP-9. Collagen I is a fibril-forming Collagen whereas Collagen IV is a network-forming Collagen. We can speculate that cells embedded in the network formed by 3D Collagen IV, but not Collagen I, can sense geometry and the external force generated by this network. Thus, in addition to the ligation of different receptors, physical properties of the 3D Collagen IV network activate cytoskeletal-triggered signaling pathways that are
distinct from those activated by 3D fibrillar Collagen I, resulting in induction of distinct MMPs.

We have demonstrated that, in contrast to MMP-1 and -3, PRL modestly inhibits MMP-2 secretion and does not affect MMP-9. PRL regulation of MMP expression is complex. PRL induces activation of MAPK pathways leading to activation of transcription factor AP-1 (activating protein 1) (Gutzman et al., 2005; Gutzman et al., 2004b). In parallel, PRL activates STAT5a and STAT5b (well-known targets of PRL/JAK2) that inhibit PRL-activated AP-1 activity. In T47D cells PRL does not alter MMP-2 expression or MMP-2 and MMP-9 activities, but reduction of STAT5 by siRNA or inhibition of STAT5 activity increases the PRL-dependent transcription/activity of AP-1 target genes such as MMP-2 (Gutzman et al., 2007; Sultan et al., 2005). In turn, overexpression of WT STAT5a inhibited MMP-2 activity. In agreement, we demonstrated that PRL inhibits MMP-2 secretion in control GFP and PAK1 WT-overexpressing cells, although not in PAK1 Y3F, suggesting that specific PRL-dependent inhibition of MMP-2 expression is dependent on PAK1 tyrosyl phosphorylation. This inhibitory effect of PRL was only detected in cells embedded in Collagen IV, but not on plastic in agreement with published data. In support of results published previously, we did not see an effect of PRL on MMP-9 expression (Gutzman et al., 2007; Sultan et al., 2005).

PAK1 facilitates MAPK signaling. PAK1 may coordinate signaling between Raf, MEK, and ERK1/2 by acting as a scaffold for these proteins (Sundberg-Smith et al., 2005). Activated PAK1 phosphorylates both Raf and MEK, enhancing their association. Following growth factor or integrin stimulation, ERK phosphorylates PAK1, resulting in
a negative feedback loop. Activated PAK1 stimulates JNK1, whereas inhibition of PAK1 activity in breast cancer cells was associated with reduction of JNK1 activity (Kumar et al., 2006). PAK1 and its upstream regulators Rac, Cdc42, and PAK-interacting exchange factor βPIX have been shown to couple to and regulate the activity of p38 MAPK (Kumar et al., 2006; Molli et al., 2009). In this study we have demonstrated that PAK1 enhances activation of JNK1/2 and accelerates activation of ERK1/2 in response to PRL. This PAK1 action depends on PRL but does not depend on 3 tyrosines (PRL has dual action on PAK1: it activates kinase activity of both PAK1 WT and PAK1 Y3F and additionally stimulates phosphorylation of 3 tyrosines on PAK1 WT by JAK2). We have assessed the effects of PRL on MAPK activation in TMX2–28 clones plated on plastic. Remarkably, we have not detected any positive effect of PRL on MMP secretion in these cells plated on plastic. However, PAK1 Y3F mutant demonstrated inhibition of MMP-3 and MMP-9 secretion as compared with control and PAK1 WT cells plated on plastic. PAK1 Y3F-dependent regulation of p38 may be responsible for this effect because PAK1 Y3F mutant stimulates activation of p38 in response to PRL, and the inhibitory effect of p38 MAPK on gene regulation has been described previously (Engel et al., 2005; Zer et al., 2007). Further experiments to assess a synergetic effect of PRL/PAK1 and 3D Collagen IV/PAK1 on MAPK’s activation are needed. How can we explain that PAK1 Y3F stimulates p38 activity much stronger that PAK1 WT in response to PRL (Figure 25A)? PAK1-dependent activation of p38 is enhanced by overexpressing of βPIX, which is a guanine nucleotide exchange factor for Rac1 and Cdc42 (Lee et al., 2001). The authors proposed a model of p38 activation in which βPIX might activate PAK1, MKK3/6, and p38 in two ways: by activating Rac1/Cdc42 through its guanine nucleotide
exchange factor activity and by interacting directly with the PAK1-regulatory N terminus. We have observed that PAK1 Y3F mutant is defective in βPIX-binding in response to PRL (Hammer et al., 2015b). Thus, overexpression of PAK1 Y3F, which cannot bind to βPIX, may instead lead to increased stimulation of Rac1/Cdc42. PAK1 Y3F can be efficiently activated by Rac1/Cdc42 (Rider et al., 2013b), leading to p38 activation and inhibition of MMP gene regulation. We still do not know whether PAK1 Y3F stimulates p38 activity in cells grown in 3D matrix to the same extent as in cells plated on plastic. We are currently investigating the role of PAK1 tyrosyl phosphorylation in p38 activation in cells embedded in 3D Collagens.

Some effects of PAK1 on the actin cytoskeleton, cell cycle arrest, and regulation of cyclin D1 promoter activity appear to be independent of PAK1 kinase activity but dependent on protein-protein interaction (Adam et al., 2000; Manser et al., 1997; Sells et al., 1997; Tao et al., 2011; Thullberg et al., 2007). It has recently been demonstrated that PAK1 phosphorylates FLNa to a greater extent when PAK1 is tyrosyl phosphorylated by JAK2 that plays important role in the PRL-dependent cell motility (Hammer et al., 2013). We suggest that tyrosyl phosphorylation of PAK1 by JAK2 creates high-affinity docking sites for binding to SH2-domain-containing proteins and alters the ability of PAK1 to find, bind, and/or phosphorylate intracellular targets, thereby amplifying the effect of PAK1 on cell function. Thus, in the presence of PRL, in addition to the kinase activity, 3 tyrosines of PAK1 become phosphorylated by JAK2 and serve as a docking site for proteins that brings an additional activity toward cell invasion. This may explain why the unphospho-Tyr-PAK1 Y3F mutant is less effective than pTyr-PAK1 WT on MMP
secretion and cell invasion following PRL treatment: it probably cannot associate with other proteins that require the phosphotyrosine docking sites for normal interaction.

PRL has a minimal (if any) effect on MMP production on cells grown on plastic. 3D Collagen IV itself stimulates MMP-1 and -3 production through WT but not Y3F PAK1, suggesting that 3 tyrosines participate in Collagen IV-triggered PAK1 regulation. PRL-induced PAK1 tyrosyl phosphorylation leads to a further increase in MMP-1 and -3 production and maximal cell invasion. The cells expressing PAK1 Y3F are still able to invade in response to PRL although to a much lesser extent that the cells expressing PAK1 WT or control cells. 3D Collagen IV also promotes MMP-2 secretion; however, this effect is PAK1-independent and modestly inhibited by PRL. We did not see an effect of PRL on MMP-9 expression whereas 3D Collagen I induces secretion of this MMP and PAK1 negatively regulates it.

These studies establish an important role for pTyr-PAK1 in breast cancer progression. PRL-dependent PAK1 phosphorylation significantly increases invasion of TMX2–28 human breast cancer cells through Matrigel, and we propose a model for PRL-dependent regulation of secretion of MMPs that integrates our findings with previous studies (Figure 39). We hypothesize that contact with 3D Collagen IV may be an important invasive stimulus for breast cancer cells. Mammary cells are normally surrounded by basement membrane, comprised mostly of type IV Collagen. In normal cells, signals from Collagen IV do not induce MMP expression. In contrast, in breast
cancer cells PRL (breast cancer cells secrete extrapituitary PRL that could behave as an autocrine growth factor (Clevenger et al., 1995; Ginsburg and Vonderhaar, 1995)) initiates the JAK2-dependent tyrosyl phosphorylation of PAK1, increasing PAK1 signaling (both through PAK1 serine/threonine kinase activity and ability to initiate
additional protein-protein interactions). Importantly, PAK1 expression is also elevated in breast cancer (Mira et al., 2000). FLNa can serve as a bridge between activated integrins/DDR and pTyr-PAK1 to integrate signals from cytokines (PRL) and the ECM (Collagen IV/integrin). FLNa regulates PRL-dependent cell migration (Hammer et al., 2013; Rider and Diakonova, 2011). PAK1 phosphorylates FLNa, leading to actin-dependent changes, and FLNa, in turn, activates PAK1 in a positive feed-back loop (Vadlamudi et al., 2002). Tyrosyl-phosphorylated PAK1 may also create additional docking sites to recruit SH2 domain-containing proteins to facilitate JAK2-dependent invasion. PAK1 activates ERK1/2, p38 MAPK, and JNK1/2, each of which can activate AP-1. Genes encoding MMP-1 and -3 have an AP-1 binding site supporting the transcription of these MMPs (but not MMP-2) after induction by PAK1. Secretion of MMP-1 and -3 is required for PRL-dependent invasion. Given the complexity of these signaling cascades, it is likely that additional signaling molecules are also involved in the modulation of MMP expression.
III. CONCLUSIONS AND PERSPECTIVES

In our studies we sought to investigate the role of tyrosyl phosphorylated PAK1 in the synergetic of E2 and PRL in breast cancer cells. We demonstrated here that tyrosines 153, 201 and 285 of PAK1 play a crucial role in its activation by E2, association of PAK1 with Gβ1γ2 and 5, Etk and βPIX, PKA activation, S305-ERα phosphorylation, and ultimately synergetic effect on cell proliferation in response to PRL+E2. Another important finding in this study is that Ser305-ERα is phosphorylated by PAK1 in response of PRL and by PKA in response to E2. We demonstrated that PRL upon binding PRLR activates JAK2. Active JAK2 tyrosyl phosphorylates and activates PAK1. PRL-dependent activated PAK1 phosphorylates S305 on ERα leading to ERα activation and regulation of breast cancer cell proliferation. We described a protein complex for PAK1 in estrogen and PRL synergistic effect on cell proliferation. E2 activates membrane-localized ERα and GPER1 leading to activation of heterotrimeric G protein. Activated G protein dissociates into α and βγ subunits which both activate several effectors. Gas stimulates AC, cAMP and subsequently activates PKA, which phosphorylates many substrates, including Ser305-ERα. Released β1γ2 and β1γ5 dimers of G protein bind to PAK1 which is tyrosyl phosphorylated by Etk. pTyr-PAK1 recruits and binds to βPIX, association with which further activates PAK1 in a positive feedback. We also demonstrated that in E2 signaling pTyr-PAK1 and PKA are reciprocally regulated.

Differentiation-inducing factors 1-3 (DIFs 1-3) that were identified in the cellular slime mold Dictyostelium discoideum, are considered anti-tumor agents because they inhibit proliferation of a variety of mammalian tumor cells in vitro. Although some
studies have shown the anti-proliferative effects of DIF-1 and DIF-3, the precise molecular mechanisms underlying the actions of DIFs are not understood. The antiproliferative effect of DIFs have been demonstrated in mammalian tumor cells including leukemia, cervical, gastric and colon tumor cells. In this study we demonstrated for the first time that DIF-3(+1) inhibits breast cancer cell proliferation. We also showed that DIF-3(+1) directly inhibits PAK1, a major participant in breast cancer signaling network. Although breast cancer cell proliferation is regulated on multiple levels, inhibition of PAK1 by DIF-3(+1) may be one of the mechanisms through which DIF-3(+1) regulate breast cancer cell proliferation.

Lastly, we demonstrated the significance of tyrosyl phosphorylated PAK1 in the regulation of breast cancer invasion, an important step preceding cancer metastasis. We have demonstrated that PAK1 phosphorylation is required for maximal invasion and that PRL and 3D Collagen IV regulate this process.

Taken together, we have demonstrated the relevance of PRL-dependent tyrosyl phosphorylated PAK1 in breast cancer progression providing: 1, a novel diagnostic/prognostic marker for the status of breast cancer in patients and 2, a novel target for therapeutics in the treatment of breast cancer.

The ERα receives stimuli from multiple ligands as had been demonstrated previously and herein by E2 and PRL to regulate both physiological and pathological processes. Increasing evidence supports a significant role of PRL in breast cancer. As described in these studies, PRL and E2 signaling pathways crosstalk contributing to breast cancer proliferation, and PRL together with Collagen promote breast cancer invasion. To date, treatment of ER positive breast cancer is through the use of selective
estrogen receptor modulators (SERMs) such as TAM and fulvestrant. The clinical limitation of the first generation SERM, tamoxifen, is that firstly only about 70% of the breast tumors are ER-positive, and the anti-tumor activity of tamoxifen is observed exclusively in tumors that express ERs. Secondly and more importantly is that almost all patients with advanced breast cancer that initially respond to tamoxifen therapy will eventually develop resistance to the therapy. The data presented here show that in the absence of E2, PRL activated PAK1 activates ERα. Such regulation of ERα and ERα target genes may explain resistance to SERMs. Treatment of patients with SERM eliminates only one pathway which may initially be effective, but once an alternative pathway begins, the whole treatment strategies becomes ineffective. It would be more beneficial and effective if a combinatorial treatment is employed, such as inhibiting ERs and PRLR. ERs can be inhibited with SERMs. PRLR can be inhibited with pure competitive PRLR antagonist hPLR-G129R, neutralizing PRL antibody, chimeric PRLR antagonist G129R-IL2 and S179D-hPRL that selectively inhibit downstream PRL targets. (Bernichtein et al., 2010; Goffin et al., 2006; Wen et al., 2014). More assessment will be required to determine if S179D-hPRL inhibit PAK1 pathways and PRL-ERα crosstalk involved in breast cancer cell proliferation. Additionally, because PAK1 regulate several downstream targets involved in cell proliferation and survival, inhibition of PAK1 in breast cancer would also be of great benefit since both E2 and PRL activate PAK1. The actions of E2 on ER negative breast cancer cells should not be ruled out. It would also be important to assess GPER1 status in the profiling of breast cancer types since GPER1 also mediate E2 actions. Lastly, but not of least importance is that the identification of markers that predict response will be extremely valuable as more targeted therapies are
developed and treatment regimens become more individualized. One of such marker
could be tyrosyl phosphorylation state of PAK1. We demonstrated in these studies that
tyrosines 153, 201 and 285 are critical amino acids that are phosphorylated on PAK1 in
response to PRL and E2. Phosphorylation of PAK1 on these residues could serve as
diagnostic markers because it would indicate that PRL or E2 or both hormones are
participating in the progression of breast cancer. In this case, appropriate treatment
regimen can be devised such as the blockade of PRL and E2 actions.

A complete understanding of how ER-regulated transcription is dysregulated in
breast cancer and how it changes with the development of resistance to endocrine therapy
will lead to improved strategies for the use of existing endocrine therapies and for the
development of better and more effective strategies for the treatment of breast cancer.
Our studies have provided more insight into ERα regulations and regulation of breast
cancer cell proliferation, however, more questions are yet to be answered.
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