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Brk/PTK6 Signaling in Normal and Cancer Cell Models

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Abstract

Breast tumor kinase (Brk), also termed PTK6, is known to function in cell-type and context-dependent processes governing normal differentiation. However, in tumors in which Brk is overexpressed, this unusual soluble tyrosine kinase is emerging as a mediator of cancer cell phenotypes, including increased proliferation, survival, and migration. Nuclear and cytoplasmic substrates phosphorylated by Brk include a collection of regulatory RNA-binding proteins, adaptor molecules that link Brk to signaling pathways generally associated with activation of growth factor receptors, and Signal Transducers and Activators of Transcription (STAT) molecules that are direct regulators of gene expression. Understanding Brk-dependent regulation of these key signaling pathways and how they influence cancer cell behavior is predicted to inform the development of improved “targeted” cancer therapies and may provide insight into ways to avoid chemo-resistance to established treatments.

Brk Identification and Expression

Breast Tumor Kinase, or Brk, was cloned from a metastatic breast tumor using a PCR-based screen for novel protein tyrosine kinases [1]. The identical protein tyrosine kinase 6 (PTK6) was independently cloned in melanoma cells as a partial sequence [2] as well as isolated as a full length clone from normal tissues [3,4]. Brk is a novel non-receptor tyrosine kinase with limited homology to the c-src kinase family. The domain structure of Brk includes SH3 (src homology 3), SH2 (src homology 2), and protein kinase domains. However, Brk lacks a consensus sequence for myristoylation present in other src family members. The *brk* gene resides on chromosome 20q 13.3–13.4 and is made up of 8 exons, which exhibit distinct boundaries relative to other family members, suggesting a functional divergence [5,6].

Brk, or the mouse homolog Sik, is normally expressed in the differentiating epithelial cells of the intestine, skin, prostate and oral cavity [3,4,7,8] and has also been detected in lymphocytes

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[9]. Levels of Brk in the normal mammary gland and in the ovary are low to undetectable. However, Brk overexpression is common in malignancies arising from these tissue types [10,11]. For example, Brk has been detected in up to 85% of human breast tumors with staining intensities correlated to histological grade [12]. Brk was also cloned from melanoma cells [2] and has been detected in cultures of metastatic melanoma cells [13]. In tumors from tissues such as colon and prostate, where Brk is expressed normally, it has been shown to be overexpressed or mislocalized [7,14], perhaps indicating altered signaling in the malignant setting. Brk has not been shown to be mutated in tumors, but appears to be frequently upregulated at the transcriptional or posttranscriptional levels [5,15,16]. Recently, Xiang *et al.* [17] reported significant co-amplification of the region of chromosome 20 where the *brk* gene (Chr 20q13.3) is localized, and chromosome 17q21–22, the location of the *erbB2* gene. In this study the *brk* gene was found amplified in 57 of 202 (28%) samples tested.

Brk Signaling

Substrates

One key to understanding the function of Brk in normal tissue homeostasis and cancer is to identify the endogenous physiologic substrates of Brk. To date a number of potential Brk substrates have been identified (Table 1; Fig. 1). These include the RNA binding proteins: Sam68, SLM-1, SLM-2, and PSF [18–21]; transcription factors: STAT3 and STAT5a/b [22, 23]; and a variety of signaling molecules: p190RhoGAP, paxillin, Akt, IRS-4, BKS/STAP-2, and KAP3A [24–29]. It is important to note that while Brk expression has been shown to induce tyrosine phosphorylation of each of these proteins, not all of these proteins have been shown to be bona fide Brk substrates *in vitro*.

Sam68 was one of the first Brk substrates identified and the most extensively studied. Derry *et al.* were the first to show that endogenous Brk and Sam68 co-localize in distinct nuclear dots, or Small Nuclear Bodies (SNBs), of cancer cell lines (Fig. 2B). Expression of Brk was also found to negatively regulate the RNA binding function of Sam68 [30]. Subsequent studies have confirmed that Brk expression negatively regulates Sam68 [20] and identified the tyrosine residues phosphorylated by Brk [18]. Interestingly, a number of other RNA binding factors have also been found to be Brk substrates [19,21]. Similar to Sam68, the Sam68-like mammalian proteins SLM-1 and SLM-2 are phosphorylated by Brk *in vitro* and Brk-induced phosphorylation negatively regulates SLM-1 and SLM-2 RNA binding function (Fig. 1A). While co-expression of SLM-1 and constitutively active Brk resulted in Brk nuclear localization [19], co-expression of Brk and the RNA binding protein PSF, resulted in PSF cytoplasmic re-localization [21].

The tyrosine phosphorylation sites have been identified for a number of Brk substrates. Brk-induced phosphorylation of paxillin was mapped to Y31 and Y118 [24]. Phosphorylation at these two tyrosines has been shown to create binding sites for CrkII, leading to Rac activation [31,32]. In a separate study, Brk was shown to phosphorylate Y1105 on p190RhoGAP-A (p190) [27]. Previous studies have shown that phosphorylation of Tyr1105 in p190 increases p190 association with p120RasGAP, leading to Rho inhibition and Ras activation [33,34]. Brk specifically phosphorylates STAT3 and STAT5a/b on tyrosines that are essential for transcriptional activation, Y705 and Y694/699 respectively [22,23]. Overall, Brk-induced phosphorylation of identified substrates has been shown to promote mitogenic and cell migration pathways (Fig. 1).

ErbB family signaling

The first reports to investigate Brk-mediated signaling events focused on the EGF pathway. Kamalati *et al.* demonstrated an interaction between Brk and the ErbB family member EGFR

and found that exogenous expression of Brk in normal mammary epithelial cells enhanced EGF-induced proliferation [35]. Subsequent studies have found that Brk can also interact with additional ErbB family members, ErbB2, ErbB3 and ErbB4 [17,36,37]. Brk overexpression in normal mammary epithelial cells enhanced the interaction between ErbB3 and PI3K, and lead to an increase in tyrosine phosphorylation of ErbB3 and PI3K/Akt signaling in response to EGF [36]. Alternatively, Brk was found to enhance ERK1/2 activation in ErbB2 overexpressing cells [17].

Additional studies have also implicated Brk downstream of ErbB signaling pathways (Fig 1C). Chen *et al.* showed that EGF induced Brk autophosphorylation, and identified paxillin as a Brk substrate. Brk-induced paxillin phosphorylation promoted Rac activation and cellular migration [24]. In support of this study, our group has shown that Brk kinase activity is activated by the ErbB receptor ligand, heregulin (HRG). We also found that HRG-induced Rac activity is dependent on Brk expression, and that Brk is required for HRG-induced activation of p38 MAPK and ERK5 [12]. Other proteins that have been shown to be phosphorylated by Brk downstream of EGF are the RNA binding proteins Sam68 and PSF [21] and p190RhoGAP [27].

Brk and Akt

Kamatlari *et al.* were the first to show that overexpression of Brk could potentiate activation of the PI3K/Akt pathway [36]. Subsequently, our group has shown that Brk and Akt interact in both breast cancer cell lines (Fig. 2A) and Cos-1 (African green monkey kidney) cells [28]. Unexpectedly, we found that WT (wild-type) but not kinase-inactive (KM) Brk inhibited Akt kinase activity in the purified Brk/Akt complex, but that this intracellular interaction was tightly regulated by EGF signaling. In Cos-1 cells, EGF stimulation resulted in dissociation of the Brk/Akt complex. EGF-induced dissociation only occurred in the presence of WT- or KM-Brk, but not when the constitutively active mutant (YF) of Brk was expressed. Interestingly, the Brk/Akt complex did not dissociate in response to EGF in T47D cells, suggesting that in these breast cancer cells, Brk behaves similarly to YF-Brk and may be constitutively active [28]. More recent studies in PTK6 knockout mice also suggest that Brk inhibits Akt in differentiating murine intestinal epithelial cells [38,39]. The small intestine of PTK6^{-/-} mice displayed elevated activation of Akt, an expanded zone of PCNA expression and increased BrdU incorporation [39]. Furthermore, when PTK6^{-/-} mice were challenged with gamma-irradiation, decreased apoptosis and increased activation of Akt was observed in the small intestine [38]. Together these studies suggest that Brk signals differently during normal development relative to cancer progression; Brk may function to limit Akt activity in normal cells or when growth factors are low, but this fails to occur in cancer cells, where Brk may somehow amplify growth factor-dependent signaling inputs to PI3K/Akt pathway activation (Fig. 2A).

STAT family signaling

Brk has also been shown to play a role in activation of STAT signaling pathways. Liu *et al.* demonstrated that Brk expression induced tyrosine phosphorylation of STAT3 and showed that immunoprecipitated Brk phosphorylated purified GST-STAT3 [23]. Furthermore, Brk expression enhanced STAT3 dependent transcription. The adapter protein BKS/STAP-2 was the first Brk interacting protein identified and shown to be phosphorylated on tyrosine residues in Brk overexpressing cells [40]. More recently, STAP-2 Tyr-250 was identified as a Brk phosphorylation site [25]. Furthermore, STAP-2 Tryr-250 phosphorylation was found to be important for Brk-induced STAT3 transcriptional activation [25]. STAT5 has also been shown to be a direct substrate of Brk, and similar to STAT3, Brk-induced phosphorylation of STAT5 potentiates STAT5 transcriptional activity (Fig. 1B) [22].

Brk kinase activity

Only a few studies have investigated the regulation of Brk kinase activity. As described above Brk kinase activity has been shown to be activated by EGF and heregulin- 1, peptide ligands that bind ErbB receptors [12,24]. IGF1 has also been shown to enhance Brk kinase activity in MCF-7 cells [29]. In this study, IRS-4, which was also shown to be a substrate of Brk, enhanced IGF1-induced Brk autophosphorylation [29]. Similar to this study, but performed in T47D cells, we also observed an IGF-induced increase in Brk autophosphorylation, which peaked at 10 minutes. Additionally, we recently showed that HGF and MSP, peptide ligands for MET and RON receptors, respectively, activate Brk in both keratinocytes and breast cancer cells (NE Castro *et al.*, unpublished). In addition to growth factor signaling, Brk has also been shown to be activated during keratinocyte differentiation. Two reports have shown that calcium rapidly activates the kinase activity of Brk/Sik in keratinocytes [41,42].

Structural and mutational studies on Brk have revealed that, while some similarities exist, key aspects of Brk kinase activity and substrate recognition are regulated differently than other Src family members. Brk kinase regulation is similar to other Src family kinases in that mutation of the C-terminal tyrosine (Y447 in Brk and Y527 in Src) to phenylalanine results in a constitutively active kinase [43]. One specific difference in the auto-regulation of kinase activity between Brk and c-Src occurs in the linker region. A conserved tryptophan in the linker region of c-Src is important for auto-inhibition of Src kinase activity. Unlike c-Src, when the conserved tryptophan (W184) in Brk is mutated, Brk kinase activity is completely abolished [44], suggesting that the linker region is important for Brk kinase activity, not auto-inhibition. Differences in Brk substrate binding have also been observed. It was found that the dissociation constant between a phosphorylated tyrosine peptide and the Brk SH2 domain is much weaker than other Src family SH2 domains [45]. It was also reported that the Brk SH3 domain plays an important role in substrate recognition [46]. Qiu *et al.* found that an SH3 binding substrate had a significantly lower binding affinity relative to a control substrate, while no difference was seen between an SH2 binding substrate and the control. The results of this study suggest that Brk SH3 domain interactions likely govern the specificity of Brk substrate phosphorylation [46].

Brk mediated biology

Brk appears to participate in a variety of signaling pathways depending on the context in which it is expressed. For example, tissue type, hormonal milieu, physiologic status (i.e. normal or cancer) and the expression levels of fellow signaling molecules may dictate the specificity of Brk action. As a result, Brk has been shown to elicit a multitude of biological effects on cells both in culture and in mouse models. In normal or non-tumorigenic tissues, Brk has largely been shown to promote cellular differentiation, apoptosis, and more recently to mediate migration/wound healing (NE Castro *et al.*, unpublished). However, in malignant settings Brk is reported to contribute to cancer progression by sensitizing cells to mitogenic signals and enhancing proliferation, anchorage-independent survival, and migration/invasion (see Table 1).

Differentiation

The major biological activity of Brk in the normal or non-malignant tissues of the skin and intestine, where it is most abundantly expressed, is to function as an inducer of cellular differentiation. The expression of Brk and mouse homologue, Sik, occurs at high levels in differentiated, nondividing epithelial cells [4,14]. Overexpression of Sik in mouse keratinocytes resulted in upregulation of a known differentiation marker [41]. Likewise, differentiation of human keratinocytes induced Brk expression which in turn required for increased expression of Keratin 10, a marker for differentiation [42]. Furthermore, Sik

knockout mice display increased growth and proliferation in the small intestine, in addition to decreased expression of a well-characterized differentiation/maturation marker, I-FABP.

Cell Cycle Progression

Brk overexpression in cells derived from normal breast, MCF10-A and HB4a cells, results in increased sensitivity to the mitogenic effects of EGF [35]. Similarly, HB4a cells displayed increased transformation potential in soft agar (anchorage independent growth) assays when exogenous WT Brk was stably expressed, but not kinase-dead (KM) Brk [35]. These data suggest that Brk expression in 'normal' cells of the breast confers a proliferative and/or survival advantage. The requirement for Brk kinase activity in these events appears to be cell-type dependent; overexpression of either WT or KM Brk induced increased EGF- or serum-stimulated proliferation of T47D cells [47] and HeLa cells (Lange, unpublished results). Likewise, in T47D breast cancer cells, loss of Brk expression via transient or stable introduction of Brk specific RNAi resulted in decreased proliferation [12,47].

While the initial reports in immortalized mammary epithelial cells suggest Brk overexpression potentiates EGF-induced proliferation, we have found that in human mammary epithelial cells (HMECs) immortalized with hTERT and SV40 early region, Brk expression inhibits EGF-induced proliferation (Fig. 3). Furthermore, the effect of Brk in these cells is dependent on Brk kinase activity. In contrast to wt and constitutively active (YF) Brk, expression of KM Brk failed to inhibit EGF-induced proliferation. One explanation for these results may be the relative expression of ErbB family members in the different cell models. As shown in Figure 3, the HMEC model expresses low levels of ErbB2, ErbB3 and ErbB4.

Recently, Brk has been shown to be co-amplified with ErbB2 in breast cancers [17]. Coexpression of Brk and ErbB2 in MCF10-A cells induces cell cycle progression by increasing Cyclin E and decreasing p27 levels. Together, Brk and ErbB2 reinitiate cell proliferation in MCF10-A 3D acinar structures leading to a transformed phenotype. Additionally, Brk expression decreases the efficacy of the ErbB2 kinase inhibitor, Lapatinib, indicating that Brk contributes to the development of resistance to current "receptor targeted" therapeutic strategies. These data suggest that the activation of Brk downstream of ErbB family members [12] should be evaluated as a potentially valuable drug target.

The participation of Brk in STAT pathways has also been demonstrated to stimulate cell growth. Liu *et al.* reported that Brk is able to induce transcriptional activation of STAT3 leading to a cooperative increase in cell proliferation [23]. Similarly, knockdown studies showed that Brk-induced activation of STAT5 is a proliferative signal in breast cancer cells [22].

Interestingly, Brk kinase activation in some non-breast cell models has been reported to decrease cell proliferation. In U2OS human osteosarcoma cells overexpression of Brk along with PSF, a Brk substrate, induces S-phase cell cycle arrest [21]. PSF phosphorylation by Brk alters its subcellular localization, decreases its RNA binding ability, and promotes PSF induced cell cycle arrest [21]. Similarly, overexpression of the Brk substrate, Sam68, decreases exogenously expressed Brk-induced proliferation in astrocytes [18]. These data illustrate that Brk is able to participate in a variety of biological signals that vary both with substrate (availability or location) and with cell type (tissue or physiologic condition).

Migration

Accumulating evidence supports a direct involvement for Brk in the processes of migration and invasion that characterize metastatic breast malignancy. An early report by Chen *et al.* placed Brk upstream of paxillin, CrkII, and Rac1 to activate migration and invasion programs in skin (A431) and breast (MDA-MB231) cancer cell lines [24]. We subsequently found that

T47D breast cancer cells are dependent upon Brk expression for their migratory response to EGF and heregulin-1. Activated Brk downstream of ErbB receptors increased Rac1, and stimulated increased p38 MAPK and Erk5 activities, leading to elevation of cyclin D1 and activation of the MEF2 transcription factor. The Brk/Rac1/p38 MAPK pathway is required for EGF or Heregulin- β 1 induced breast cancer cell migration [12]. In BT20 breast cancer cells expression of constitutively active Brk induced cells to migrate in transwell assays via phosphorylation of KAP3A, while KM Brk caused little more than control level migration [26]. Recently, Brk-induced phosphorylation of p190RhoGAP-A was found to play a role in EGF-induced motility and proliferation in mouse embryonic fibroblasts (MEF) cells and, migration, invasion, and proliferation in MDA-MB231 breast cancer cells [27]. Interestingly, HGF-induced migration of T47D and MDA-MB231 cells is dependent upon Brk and ERK5 complexes, but can occur independently of Brk kinase activity (NE Castro *et al.*, unpublished). These data suggest that Brk employs a variety of signaling pathways and complex mechanisms to induce cell migration, but support the notion that in a subset of tumors, Brk may primarily act to promote cellular migration, invasive behavior and metastasis.

Angiogenesis

Wt Brk, but not KM Brk, has also been shown to promote angiogenesis in response to the chemokine, osteopontin (OPN). MDA-MB-231 cells respond to osteopontin by activating Brk, NF- κ B, and ATF-4 signaling which leads to paracrine and juxtacrine signaling inducing VEGF dependent endothelial cell and breast cancer cell motility [48]. These data were validated *in vivo* using a matrigel plug assay which showed that OPN promoted angiogenesis and tumor cell proliferation in a VEGF dependent manner [48].

Apoptosis and Autophagy

Brk kinase activity has been reported to play competing roles in cell death pathways, dependent upon the context of its expression. In breast cancer cells, *oncogenic* Brk functions to protect cells from autophagic induced death under anchorage independent conditions [49], whereas in normal (non-malignant) tissues *proto-oncogenic* Brk sensitizes epithelial cells of the skin and intestine to apoptosis induced by serum deprivation and DNA damage [50]. These findings were confirmed *in vivo* in the intestine of Sik KO-mice; following total body irradiation Brk expression was induced and subsequently promoted cellular apoptosis in wt but not KO mice [38].

Summary

Together, the findings discussed herein suggest a divergent role for Brk as a regulatory *proto-oncogene* in selected tissues compared to the tumor-promoting activities that *oncogenic* Brk clearly displays in malignant settings, where it appears to promiscuously use growth factor (erbB, IGF, MET) receptor-directed pathways to contribute to disease progression. Although important roles for Brk kinase activity (i.e. Brk-mediated phosphorylation events) have been clearly shown to mediate effects on cell biology in some settings, scaffolding activities of Brk also appear to sufficiently regulate biology in other contexts. Going forward, it will be important to fully characterize the role of Brk substrate molecules and interacting proteins in order to discern the importance of Brk kinase activity relative to Brk domain (i.e. SH2 and SH3) structure as potential contributors to aberrant cancer cell biologies. Further studies on the regulation of Brk expression as early tumors progress may provide additional clues to how cancer cells adapt in order to evade host defenses and escape clinical interventions. Brk expression may inform therapy decisions. For example, Brk (as a mediator of apoptosis) may be a liability for cells exposed to radiation or classical chemotherapies aimed at inducing DNA damage [50]. On the other hand, Brk (as a downstream effector of erbB2) may provide tumors with an advantage over newer “targeted” therapies (i.e. herceptin and other antibodies) aimed

at the level of cell surface growth factor receptors [17]. Thus, cell (tumor type and stage) specific mechanisms of Brk action are vital considerations as we continue to navigate the era of “rational drug design” characterized by the use of combination and/or sequential delivery of cancer therapies.

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Figure 1. Brk acts as a mediator for multiple signaling pathways

A. Tyrosine phosphorylation by Brk decreases Sam68 (as well as SLM-1 and SLM-2, not shown) RNA binding activity. **B.** Brk phosphorylation enhances Stat3 and Stat5 transcriptional activity. **C.** Brk signaling downstream of ErbB receptors activates multiple signaling pathways which can lead to changes in the gene programs expressed by cells.

Figure 2.

A. Co-localization of Brk and Akt. MCF-7 cells growing on coverslips were treated with EGF (5 min) and Brk (green) and Akt (red) were stained using fluorescent-tagged antibodies. Each molecule stained throughout cells, but co-localization (yellow) intensified to membrane regions following EGF stimulation. **B.** Redistribution of Sam68 proteins in breast cancer cells. Sam68 was predominantly nuclear in resting cells, but dramatically became highly concentrated into SNB-like structures within the nucleus upon EGF treatment. Antibody controls indicated Brk-specific or Sam68-specific staining.

Figure 3.

A. Western blot analysis for expression of Brk, ErbB1, ErbB2, ErbB3, ErbB4 and Actin. Total cell lysates were collected from asynchronous HeLa, T47D and HMEC cell lines. Brk overexpressing HMECs were generated by infecting SV40-LT/hTERT immortalized parental HMECs (P) with retrovirus encoding wild-type (WT), kinase inactive (KM), or constitutively active (YF) Brk and then selecting for infected cells with zeomycin. Parental HMECs were also infected with retrovirus containing the vector control (V), pBabeZeo. B. Brk kinase assay from HMEC cell lines described in A. Brk was immunoprecipitated from HMECs with a Brk specific antibody. Immunoprecipitated Brk was then incubated with recombinant Sam68 in the presence of ATP. Brk and Sam68 were then resolved by SDS-PAGE and western blotting for tyrosine phosphorylated proteins (4G10) was performed. The amount of Brk immunoprecipitated and present in the total cell lysate was determined by western blotting. C. Brk expression inhibits EGF-induced proliferation in HMECs. Representative MTT assay from HMEC cell lines described in A. Cells were plated at 1×10^4 cells per well in a 24-well plate in the absence of growth factors. The following day (Day 0) cells were treated with 10 ng/mL EGF or left untreated. MTT readings were obtained on days 0, 3, and 6. All data points were divided by the Day 0 reading to give a fold increase. Error bars represent standard deviation.

Brk Substrates

Table 1

The left hand column lists all proteins that have been identified as potential Brk substrates. The column labeled “Function” refers to the intracellular function of the Brk substrate. “Signaling Pathway” refers to the signaling pathway the substrate and Brk have been shown to play a role in, while “Biology” refers to the biological significance of Brk-induced phosphorylation of the substrate. The tyrosine residues found to be phosphorylated by Brk are indicated in the column labeled “Phosphorylation Sites” and the references for the data summarized in the table are in the right hand column.

Brk Substrate	Function	Signaling Pathway	Biology	Phosphorylation sites	References
Sam68	RNA binding protein	EGF	Cell Cycle	Y345, Y440, Y434	[18,20,30]
SLM-1/SLM-2	RNA binding protein				[19]
PSF	RNA binding protein	EGF	Cell Cycle	C-terminus	[21]
STAT3	Transcription factor		Cell Cycle	Y705	[23]
STAT5a/b	Transcription factor		Cell Cycle	Y694/Y699	[22]
Paxillin	Adapter protein	EGF	Migration	Y31, Y118	[24]
p190RhoGAP	GTPase activating protein		Migration	Y1109	[27]
KAP3A	Subunit of kinesin-2 complex		Migration	C-terminus	[26]
BKS/STAP-2	Adapter protein	Stat3 activation		Y250	[25,40]
Akt	Serine/threonine kinase	EGF,	Intestinal epithelial cell cycle and differentiation		[28,38,39]
IRS-4	Adapter protein	IGF			[29]