Breast Tumor Kinase (Brk/PTK6) Is a Mediator of Hypoxia-Associated Breast Cancer Progression

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Abstract

Basal-type triple-negative breast cancers (TNBC) are aggressive and difficult to treat relative to luminal-type breast cancers. TNBC often express abundant Met receptors and are enriched for transcriptional targets regulated by hypoxia-inducible factor-1α (HIF-1α), which independently predict cancer relapse and increased risk of metastasis. Brk/PTK6 is a critical downstream effector of Met signaling and is required for hepatocyte growth factor (HGF)-induced cell migration. Herein, we examined the regulation of Brk by HIFs in TNBC in vitro and in vivo. Brk mRNA and protein levels are upregulated strongly in vitro by hypoxia, low glucose, and reactive oxygen species. In HIF-silenced cells, Brk expression relied upon both HIF-1α and HIF-2α, which we found to regulate BRK transcription directly. HIF-1α/2α silencing in MDA-MB-231 cells diminished xenograft growth and Brk reexpression reversed this effect. These findings were pursued in vivo by crossing WAP-Brk (FVB) transgenic mice into the METMut knockin (FVB) model. In this setting, Brk expression augmented METMut-induced mammary tumor formation and metastasis. Unexpectedly, tumors arising in either METMut or WAP-Brk × METMut mice expressed abundant levels of Sik, the mouse homolog of Brk, which conferred increased tumor formation and decreased survival. Taken together, our results identify HIF-1α/2α as novel regulators of Brk expression and suggest that Brk is a key mediator of hypoxia-induced breast cancer progression. Targeting Brk expression or activity may provide an effective means to block the progression of aggressive breast cancers. Cancer Res; 73(18); 5810–20. ©2013 AACR.

Introduction

Breast tumor kinase (Brk), also known as PTK6, is a soluble protein tyrosine kinase typically expressed in differentiated epithelial cells of the skin and gastrointestinal tract (1). Although Brk is not found in normal mammary tissue, it is aberrantly expressed in up to 86% of breast tumors, with the highest levels in advanced tumors (2–4). Other cancers, such as melanoma, lymphoma, ovarian, prostate, and colon cancer also exhibit overexpressed and/or mislocalized Brk (reviewed in ref. 5).

Brk contains N-terminal src homology 2 (SH2), src homology 3 (SH3), and C-terminal kinase domains. It is distantly related to Src family kinases, as they share 56% homology within the kinase domain (6). Brk lacks a myristoylation site, and is present in both the cytoplasm and the nucleus. Many Brk substrates, both cytoplasmic and nuclear, have important functions in cancer, including STAT molecules, Akt, and Sam68 (reviewed in ref. 5). Brk is activated downstream of ErbB family receptors and Met receptors and is required for EGF-, heregulin-, and hepatocyte growth factor (HGF)–enhanced cell migration (7–9). Although Brk and ErbB2 have distinct gene loci, they are coamplified in some breast cancers (10). Brk expression in ErbB2-induced tumors correlates with shorter latency and resistance to the ErbB2 inhibitor, lapatinib (10). Moreover, elevation of Brk expression in breast cancer cells confers resistance to the EGF receptor (EGFR)–blocking antibody, cetuximab, by inhibiting EGFR degradation (11). Brk also mediates anchorage-independent growth in breast cancer cells through modulation of the insulin-like growth factor (IGF) receptor (12). Although significant advancements have been made toward understanding the mechanisms of Brk signaling (5), little is known about the regulation of Brk expression in breast cancers.

Hypoxia-inducible factors (HIF) are the principal mediators of transcriptional responses to cellular hypoxia (13). Hypoxia-inducible factors (HIF-1 and HIF-2) are heterodimers of two oxygen-regulated subunits, HIF-1α or HIF-2α and HIF-1β.
HIF-1β is constitutively expressed, whereas HIFα subunits are continually degraded through the ubiquitin pathway under normal oxygen tensions (normoxia). In response to hypoxia, HIFα subunits are stabilized and translocate to the nucleus, where they heterodimerize with HIF-1β. HIF transcription factors recognize core hypoxia-response elements (HRE) within enhancer regions of target genes (14, 15), and act as master regulators of many cellular functions relevant to cancer progression, including angiogenesis, glucose metabolism, and tumor growth and metastasis (13). Indeed, HIF-1α is overexpressed in many human cancers (reviewed in ref. 16), and overexpression in breast tumors predicts relapse and indicates a higher risk of metastasis (17). HIF-1α levels are significantly higher in invasive and poorly differentiated breast cancers as compared with well-differentiated cancers (18–20). Specifically, increased levels of HIF-1α mRNA and the core hypoxic transcriptional response are associated with hormone receptor–negative breast cancers (19, 21).

Breast tumors lacking estrogen receptor (ER), progesterone receptor (PR), and HER2, termed triple-negative breast cancers (TNBC), are typically more aggressive relative to ER/PR/HER2–positive tumors. TNBCs largely fall into the basal and claudin-low molecular subtypes and have a worse prognosis relative to luminal breast cancers (reviewed in ref. 22), in part, because these patients are not candidates for targeted therapies that block ER and HER2. Patients with TNBC are treated with systemic chemotherapies that include cytoskeletal- or DNA-blocking agents, which can be effective, but fail to specifically target the unknown and presumably diverse molecular drivers of cancer metastasis. As Brk is aberrantly expressed in both luminal and TNBC subtypes, but is not found in the normal mammary tissue, it is an attractive candidate for selective targeting of invasive breast cancer cells.

Herein, we examined the mechanism of Brk induction in breast cancer, with focus on TNBC/basal-type breast cancers abundantly expressing both MET and HIF target genes. We hypothesized that Brk, a known mediator of Met signaling and stress-activated kinase pathways, is upregulated in response to cellular hypoxia, thereby promoting cancer cell survival, cell motility, and metastasis.

Materials and Methods

Cell culture
MDA-MB-231 cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM; HyClone Thermo Scientific) without pyruvate supplemented with 10% FBS (Gibco, Invitrogen) and 1% penicillin/streptomycin. Stable knockdown of HIF1A, HIF2A, or both genes in MDA-MB-231 cells was generated by transduction as previously described (see Supplementary Methods; ref. 20). MDA-MB-231 shControl, shHIF1A, and shHIF2A cells were supplemented with 4 μg/mL puromycin, and shHIF1A/2A cells were supplemented with 8 μg/mL puromycin and 2 mg/mL hygromycin and authenticated on April 11, 2013 by SoftGenetics LLC or DDC Medical and results were compared with the American Type Culture Collection short-tandem repeat (STR) database. Cells were maintained in 5% CO2 at 21% O2 (normoxia, ambient air) or at 1% to 2% O2 (hypoxia).

Cell proliferation assay
Proliferation was measured via MTT assay as previously described (23). MDA-MB-231 cells were plated at 2.5 × 104 cells per well in 24-well plates.

Protein extraction
Patient-derived xenograft tissue fragments maintained by the HCI breast tumor bank resource (24) were obtained (Supplementary Table S1). High-salt enriched whole-cell lysates (HS-WCE) were prepared from HCI tumors or MDA-MB-231 tumors as previously described (25). Whole-cell lysates from cultured cells were isolated as described in ref. 26. Additional human tumor specimens were obtained from the University of Minnesota Biological Materials Procurement Network (BioNet) and histologically subtyped and processed for protein expression as previously reported (23).

Immunoblotting
Proteins were resolved on 7.5% SDS-PAGE or 3% to 8% Tris-Glycine gels, transferred to polyvinylidene difluoride (PVDF) membrane and probed with primary antibodies: HIF-1α (Novus Biologicals; NB100-134 or NB-100-479), HIF-2α (Novus Biologicals; NB100-122), p38 (Cell Signaling Technology; 9212), vinculin (Sigma; V9131), actin (Sigma; A4700), Brk (Santa Cruz Biotechnology; sc-1188), Survivin (Santa Cruz Biotechnology; sc-96), or β-tubulin (AbCam; 6046). Secondary horseradish peroxidase–conjugated antibodies (Bio-Rad or Santa Cruz Biotechnology) were visualized with SuperSignal West Pico or Millipore enhanced chemiluminescence (ECL) substrate. Representative images of triplicate experiments are shown. Densitometry was conducted via ImageJ analysis and normalized to the loading control.

Quantitative real-time PCR
Quantitative real-time PCR (qRT-PCR) assays were conducted as previously described (26). Briefly, cells were plated at 2.5 × 104 cells per well in 6-well plates and cultured at normoxia for 32 hours, then transferred to hypoxia (1% O2) for 24 hours or left at normoxia. Target gene expression was normalized to the expression of internal control, TATA-binding protein (TBP).

Chromatin immunoprecipitation assays
Chromatin immunoprecipitation (ChIP) assays were conducted as previously described (26). Briefly, MDA-MB-231 cells were plated at a density of 3 × 106 cells per 15-cm cell culture dish and cultured at normoxia for 32 hours, then cultured at normoxia or 1% O2 for 24 hours. Lysates were immunoprecipitated overnight (18 hours) with 2 μg of HIF-1α antibody, HIF-2α, and RNA polymerase II (Covance; 8WG16) or an equal amount of rabbit immunoglobulin G (IgG). Negative control rabbit IgG was immunoprecipitated from MDA-MB-231 cultured at hypoxia for 24 hours.

Transgenic mice and generation of tumors in NOD/seid/gamma recipients
MMTV-PyMT–HIF-1 wild-type (WT) and knockout mammary tumors were generated as described in ref. 25. WAP-Brk...
transgenic mice (4) and Met-mutant knockin mice were generated as described previously (27), monitored daily for tumor development and euthanized when tumor volume approached 1 cm\(^3\). Cultured MDA-MB-231 cells were dissociated into single cells and diluted 1:1 (v/v) with growth factor–reduced Matrigel—Hank’s balanced salt solution (HBSS) at 250,000 cells per 10 μL. Cells were injected into the right inguinal mammary fat pad of 3- to 6-week-old female NOD/scid/ILR2γ (NSG) recipients. Recipients were palpated up to two times per week, and tumor volumes were calculated by caliper measurement as described previously (28). At experimental endpoint, tumor wet weight was also measured.

**Immunostaining**

Immunohistochemistry of HIF-1 WT and knockout mammary formalin-fixed paraffin-embedded (FFPE) tumor sections was conducted as previously described (4). Briefly, tissues were incubated 1 hour at room temperature with serum-free protein block (Dako; X0909), then incubated overnight at 4°C with primary anti-Sik antibody [1:1,000 in Dako Antibody diluent parafﬁnembedded (FFPE) tumor sections (5–7 μm) were antigen retrieved using 1× citrate buffer, stained with Sik primary antibody (sc-916) at 1:50 dilution overnight at room temperature, followed by Alexa Flour-594 (Invitrogen) secondary and mounted with VECTASHIELD (Vector Laboratories, Inc.).

**Kaplan–Meier curves**

Survival analysis was conducted using the van’t Veer microarray dataset downloaded from Rosetta Inpharmatics (http://bioinformatics.nki.nl/data/van-t-Veer_Nature_2002/). Normalized Brk expression values were divided into four quartiles: 75 tumors with high Brk expression (>0.074) and 76 tumors with lowest Brk (<−0.036) expression. The y-axis (probability) is defined as the frequency of survival. Data were analyzed using the survival package within WinSTAT for Excel.

**Statistical analysis**

Results are presented as means ± SEM. Statistical significance for qRT-PCR assays was determined using unpaired Student t tests. Tumor xenograft growth significance over time was determined via two-way ANOVA with Bonferroni correction. Tumor latency was analyzed using Kaplan–Meier methodology and curves compared using the Mantel–Cox log-rank test. Brk mRNA levels were assessed using The Cancer Genome Atlas (TCGA) data.

**Results**

**Brk is upregulated in response to hypoxia and cellular stress**

Numerous studies have shown Brk overexpression in breast and other cancers relative to normal control tissues (5). Data from TCGA were analyzed via Oncomine to compare the levels of Brk mRNA expression in a large number of high-quality samples representing both invasive ductal and invasive lobular breast cancer versus normal breast tissues. Interestingly, independent of tumor subtype, Brk expression was significantly increased in both invasive ductal carcinoma (\(P = 1.50E^{-35}\)) and invasive lobular carcinoma (\(P = 3.35E^{-10}\)), relative to normal breast tissue samples (Fig. 1A). To investigate Brk expression levels specifically in basal/TNBCs, we collected a panel of TNBC cell lines and tumors. Human tumor samples were histologically scored and processed as previously described (23). We observed a range of Brk expression by Western blot analysis in cell lines and tumor samples (Fig. 1B). In addition, Brk expression was assayed in a subset of previously described (24) xenografted tumors maintained in the Huntsman Cancer Institute (HCI; Salt Lake City, UT) breast tumor tissue bank. All HCI TNBC and luminal B (HER2\(^{+}\) tumors (Fig. 1C) were highly enriched in both HER2\(^{+}\) tumors (Fig. 1C; Supplementary Table S1). In addition, both HIF-1α and HIF-2α proteins were highly enriched in the TNBC samples relative to HER2 or steroid hormone
receptor-positive tumors. These data confirm that Brk, HIF-1α, and HIF-2α are coexpressed in human breast carcinomas, particularly in TNBCs.

Multiple mechanisms of Brk upregulation exist (10, 29). We hypothesized that Brk expression may also be induced upon cellular stress. TNBC cells were exposed to various stresses, including hypoxia, and we examined Brk protein and mRNA expression. MDA-MB-231 breast cancer cells were subjected to mild hypoxic conditions (2% O2) and harvested for Western blot analysis. HIF-1α protein was upregulated following exposure of MDA-MB-231 cells to low oxygen for 4 to 30 hours relative to cells incubated in normoxic conditions (Fig. 2A). HIF-1α protein levels peaked at approximately 6 hours of hypoxia, followed by decreased but sustained expression out to 30 hours. Interestingly, Brk levels increased at 6 hours of hypoxia compared with normoxic controls, coinciding with the peak of HIF-1α protein expression. Elevated protein levels were maintained out to 30 hours. The same experiment was carried out in nontumorigenic immortalized MCF10A cells, previously reported to be Brk-null (Fig. 2B; ref. 30), as well as ER+/PR+/MCF7 (Supplementary Fig. S1A) and T47D breast cancer cells (data not shown). Following 24 hours of hypoxia exposure, Brk levels were consistently increased in all cell lines.

To assess the transcriptional regulation of Brk during hypoxia, we cultured Hs578T and MDA-MB-231 cells for 24 hours in 1% O2 and examined Brk mRNA levels by qRT-PCR. Brk transcript levels increased significantly at hypoxia compared with normoxia, in both TNBC cell lines (Fig. 2C). Levels of VEGF, a known HIF-1 target gene, also significantly increased in both cell lines. Interestingly, transcript levels of ERBB2 and Met receptor (MET), growth factor receptors known to activate Brk signaling, were also increased significantly by hypoxia in MDA-MB-231 cells. Similar results were observed in MCF7 breast cancer cells (Supplementary Fig. S1B).

The above results suggest that Brk induction may be characteristic of more universal responses to cellular stresses that also input to HIF-1α (31). We therefore assessed the levels of Brk expression in response to increasing concentrations of reactive oxygen species (ROS) by treatment with hydrogen peroxide (H2O2) and following glucose deprivation. When MDA-MB-231 cells were exposed to H2O2 (0–100 μmol/L), Brk and HIF-1α protein levels were induced or stabilized, respectively, in a dose-dependent manner (Fig. 2D). A similar response occurred when cells were exposed to media containing lowered glucose (1 g/L) as compared with base DMEM-Hi media (4.5 g/L; Fig. 2E). These data, indicating Brk induction by multiple cell stress pathways, suggest a mechanism for coordinate regulation of downstream signaling in response to HIF activation.

**Brk is a novel, direct HIF transcriptional target gene**

The Brk promoter contains multiple potential HREs within 20 kb of the transcriptional start site (TSS; Fig. 3A). To examine HIFα recruitment in these regions, we conducted ChIP assays with MDA-MB-231 cells cultured at normoxia or hypoxia for 24 hours. We observed that HIF-1α and HIF-2α were robustly
recruited to HRE 1 located 1.5 kb upstream of the Brk TSS at hypoxia compared with normoxia (Fig. 3B). As a functional correlate of transcriptional activity associated with this HRE, we assessed the recruitment of RNA polymerase II to this region (HRE 1) and observed robust recruitment of this enzyme following exposure to hypoxia (Fig. 3B). These data suggest that HIF-containing transcriptional complexes present at HRE 1 in hypoxia are active. Essentially identical results (i.e., hypoxia-regulated recruitment of HIF-1α, HIF-2α, and Pol II) were obtained for HREs 2–5 (Supplementary Fig. S2). HIF-1α recruitment to the VEGF promoter (Fig. 3C) was included as a positive control (32). The first intron in the transcriptionally inactive hemoglobin B (HBB) gene (33) served as a negative control (Fig. 3D). These data, strongly implicate HIFs in upregulation of Brk mRNA under conditions of cellular stress in MDA-MB-231 cells.

To determine whether HIF-1α was required for the induction of Brk in aggressive, metastatic mammary tumors, we first examined the expression of Sik, the mouse homolog of Brk, in end-stage HIF-1 WT or knockout mouse mammary tumors initiated by expression of the mouse mammary virus-driven polyoma middle T oncoprotein (MMTV-PyMT) transgene, as previously described (25). Colorimetric immunohistochemistry staining for Sik revealed a substantial loss of Sik protein in HIF-1 knockout PyMT tumours relative to HIF-1 WT tumours (Fig. 4A). These data strongly implicate HIFs in upregulation of Brk mRNA under conditions of cellular stress in MDA-MB-231 cells.

Figure 3. Brk is a direct HIF target gene. A, schematic representation of HREs in the proximal Brk promoter that were assessed for HIF-1α/2α recruitment. B, MDA-MB-231 cells were cultured at normoxia or hypoxia (1% O2) for 24 hours. ChIP assays with HIF-1α, HIF-2α, or RNA pol II antibodies and qRT-PCR were conducted on the isolated DNA to determine HIF and pol II recruitment to HRE 1. Negative control isotype-matched IgG controls were conducted on MDA-MB-231 cells cultured at hypoxia for 24 hours. ChIP for HIF-1 was conducted for the VEGF promoter (C) or the negative control HBB intron (D). Representative examples from triplicate experiments are shown.

Despite dramatic reduction in HIF-1 knockout PyMT-tumors, Sik protein was still weakly detected (Fig. 4). Notably, HIF-1α and HIF-2α have overlapping transcriptional targets (20, 35–37). Therefore, we tested the dependence of Brk expression on HIFα molecules in human breast cancer cells cultured under hypoxic conditions. MDA-MB-231 cells expressing empty vector (shControl), HIF1A short hairpin RNA (shRNA), HIF2A shRNA, or both HIF1A and HIF2A shRNAs [double-knockdown (DKD)] were cultured at normoxia or 1% O2 for 6 or 24 hours. As expected, HIF-1α and HIF-2α protein levels were increased at 6 and 24 hours in shControl cells cultured at hypoxia; target gene expression was greatly reduced in HIF1α and HIF2α shRNA-expressing cells (Fig. 5A). We observed a substantial hypoxia-induced increase in HIF-2α protein expression in cells expressing shHIF1A relative to shControl. The upregulation of HIF-2α in response to efficient HIF1A knockdown was previously shown in MCF-7 cells (20). Importantly, only DKD cells completely lacked both HIF molecules (Fig. 5A). Brk mRNA was significantly decreased in localized in the tumor epithelium and stroma. A no primary-antibody control showed that immunoreactivity was entirely due to the primary Sik antibody (Fig. 4A); the specificity of Sik antisera was shown previously (34). In addition, four HIF-1 WT and four HIF-1 knockout PyMT tumors were assessed for Sik protein levels by Western blotting. We observed a marked reduction in Sik protein in HIF-1 knockout tumors relative to HIF-1 WT tumors (Fig. 4C). Therefore, HIF-1α is required for robust expression of Sik in PyMT-mouse mammary tumors.

To determine whether HIF-1α was required for the induction of Brk in aggressive, metastatic mammary tumors, we first examined the expression of Sik, the mouse homolog of Brk, in end-stage HIF-1 WT or knockout mouse mammary tumors initiated by expression of the mouse mammary virus-driven polyoma middle T oncoprotein (MMTV-PyMT) transgene, as previously described (25). Colorimetric immunohistochemistry staining for Sik revealed a substantial loss of Sik protein in HIF-1 knockout PyMT tumours relative to HIF-1 WT tumours (Fig. 4A). These data strongly implicate HIFs in upregulation of Brk mRNA under conditions of cellular stress in MDA-MB-231 cells.
MDA-MB-231 cells expressing either shHIF1A or shHIF2A relative to shControl cells, but significantly induced by hypoxia relative to normoxia (Fig. 5B). However, when both HIF1A and HIF2A were simultaneously knocked down, we observed an almost complete ablation of Brk transcript and protein expression (Fig. 5B inset). Similar results were observed in MCF7 cells (Supplementary Fig. S1C). These data suggest that when expression of either HIF-1α or HIF-2α is lost, the other HIFα subunit may compensate to induce Brk expression, consistent with the regulation of numerous other HIF-target genes (20).

**Ectopic Brk expression enhances growth of HIF1A/ HIF2A knockout tumors in vivo**

Previous studies showed that tumors derived from MDA-MB-231 cells that lack HIF-1α and HIF-2α displayed significantly decreased growth relative to WT controls (38–40). As Brk knockdown in breast cancer cells has been shown to inhibit proliferation and block cell migration in vitro (5) as well as decrease xenograft tumor size in vivo (41), we sought to determine whether Brk overexpression could compensate for decreased tumor growth caused by loss of HIF-1 and -2 activity (i.e., a model where endogenous Brk is not HIF-induced). Thus, MDA-MB-231 DKD cells were retrovirally infected with a pFB-neo-WT-Brk overexpression construct. Western blot analysis confirmed that knockdown of HIF1A and HIF2A was maintained in DKD cells (Supplementary Fig. S3A), and the cells were thus unable to induce endogenous Brk (Supplementary Fig. S3B). In contrast, DKD cells engineered to overexpress WT-Brk exhibited constitutive Brk expression, as expected (Supplementary Fig. S3B). Consistent with previously published in vivo results (38–40), DKD cells grew significantly less than shControl cells via in vitro MTT assays. This growth defect was recovered (days 1–3) and then surpassed (day 5) upon reexpression of WT-Brk (Fig. 6A). These cells (shControl, DKD, and DKD + Brk) were used to establish orthotopic mammary tumors in NOD/scid/ILR female recipients (n = 9 mice/cohort). Xeno-grafts were palpated and tumor growth was measured with digital calipers as previously described (28). In agreement with previous reports (38–40), knockdown of both HIF1A and HIF2A significantly decreased breast cancer cell growth in vivo relative to shControl cells at day 38; 780.6 ± 98.4 mm³ versus 558.0 ± 36.0 mm³, respectively (Fig. 6B and C).

Surprisingly, DKD cells constitutively expressing exogenous Brk (DKD + Brk) exhibited robust in vivo xenograft growth that not only reversed the HIF DKD growth phenotype (P < 0.05 at
lymph nodes was con
ropsy or hemotoxylin and eosin (H&E) analysis (0 of 5 mice/
3 inches, whereas no mice bearing DKD or shControl
þ
DKD
lesions. We observed that 40% of the mice transplanted with
regions were necropsied to detect the presence of lymph node
mental time course (data not shown).

Figure 5. Brk mRNA and protein expression are ablated upon loss of both
HIF-1α and HIF-2α. A, MDA-MB-231 cells expressing shControl, shHIF1A, shHIF2A, or DKD were cultured at normoxia or hypoxia (1% O2) for 6 or 24 hours. Lysates were analyzed by Western blotting with antibodies to HIF-1α or HIF-2α or β-tubulin (loading control). The asterisk indicates a cross-reactive band and the arrow indicates HIF-2α. B, MDA-
MB-231 cells expressing shControl, shHIF1A, shHIF2A, or DKD were cultured at normoxia or hypoxia (1% O2) for 24 hours. Protein and mRNA were isolated and mRNA was analyzed by qRT-PCR after normalization to TBP. Inset, Western blot analysis for Brk and ERK1/2 (loading control).

day 35 and P < 0.001 at day 38; day 38 mean tumor volume 921.9 ± 88.5 mm^3 vs. 558.0 ± 36.0 mm^3, respectively), but exceeded that of shControl tumors at day 38 posttransplant (Fig. 6B; 921.9 ± 88.5 mm^3 vs. 780.6 ± 98.4 mm^3, respectively; P < 0.05). These data indicate that constitutive Brk expression is sufficient to recover the diminished growth phenotype of HIF DKD cells. In addition, comparison of mean evo vivo tumor mass confirmed that the shControl xenograft tumors were significantly larger than DKD tumors (Fig. 6C; P = 0.05). Similarly, DKD + Brk xenograft tumors were significantly larger than DKD tumors as determined by comparison of tumor wet weight (P < 0.01). Western blotting of end-stage tumors confirmed the maintenance of HIF knockdown over the experimental time course (data not shown).

These results were independently repeated wherein axillary regions were necropsied to detect the presence of lymph node lesions. We observed that 40% of the mice transplanted with DKD + Brk xenograft tumors harbored lymph node macro-metastases (2 of 5 mice), with node masses greater than 2 mm in diameter, whereas no mice bearing DKD or shControl xenograft tumors exhibited overt node involvement by necro-

Brk increases the appearance of basal-like mammary tumors in METMut mice

Our recent work showed that Brk signals downstream of Met receptors to increase HGF-driven malignant processes in evo vitro (8). High Met receptor levels are also strongly correlated with TNBC and basal-like breast cancers (27). Therefore, we sought to determine the effects of Brk-dependent signaling on mam-
mary tumor development in a well-characterized mouse model of human breast cancer. To generate mammary tumors, WAP-
Brk (FVB) transgenic mice were crossed with METMut knockin (FVB) mice, which express a constitutively active Met receptor and mimic the pathologic features of human basal-like breast cancer (4, 27). As the Met receptor is known to be an upstream activator of Brk, mammary epithelial cells derived from WAP-
Brk × METMut mice are predicted to have constitutive activi-

Following continuous pregnancies (n = 3) to activate mam-
mary gland–specific WAP-driven Brk transgene expression, groups of age-matched, multiparous female mice (n = 17 mice/ cohort; WT, Brk alone, METMut alone, or Brk × METMut) were monitored for tumor formation weekly and sacrificed once tumors grew to a volume of approximately 1 cm². Mice in the WT and WAP-Brk (i.e., nonactivated Brk) cohorts failed to develop mammary tumors over the experimental time course (Fig. 7A). As predicted (27), nearly 100% of METMut mice developed mammary tumors by day 700. However, increased tumor formation and decreased overall tumor-free survival were observed in WAP-Brk × METMut mice relative to METMut mice (log-rank test, P = 0.03; Fig. 7A). The median survival of WAP-Brk × METMut mice was 228 days, whereas median survival of METMut was 368 days. We collected tissues (lung, liver, brain, and ovaries) from mice upon necropsy for analysis of potential (latent) metastatic events (Table 1). Importantly, Brk × METMut mice developed more (20%) metastases relative to METMut mice (12%; Fig. 7B; Table 1). Notably, metastases were observed in mice that survived (i.e., were not euthanized because of primary tumor size) at least 275 days; of these, 3 of 6 (50%) Brk × METMut mice developed distant metastases, whereas 2 of 15 (13%) METMut mice developed distant metastas-

Most solid tumors are hypoxic. Our evo vitro studies related to
hypoxia regulation of Brk expression (Figs. 2 and 3) suggested that en
dogenous Sik expression may be induced during the course of tumor progression in METMut mice. Therefore, all tumors extracted from mice were screened for Sik mRNA expression via qRT-PCR. Notably, when data were stratified by Sik expression alone, regardless of Brk transgene genotype, there was a strong association between Sik positivity and decreased tumor-free survival (log-rank test, P = 0.0006; Fig. 7C). Mice with Sik-positive tumors had a median survival of 221 days, whereas mice with Sik-negative tumors had a median survival of 397.5 days. Overall, the decreased latency correlated with significantly higher levels of Sik in tumors (Fig. 7C and D), suggesting that Sik expression accelerates tumor development and progression.
To further support these findings as relevant to human tumors, we stratified 151 primary human breast tumor samples according to Brk mRNA expression (42). High Brk expression correlated significantly with both decreased overall survival (Fig. 7E) and decreased metastasis-free survival (Fig. 7F). These data suggest that the WAP-Brk/C2METMut mice model human disease with high Brk expression. These mice may provide a useful model system for preclinical testing of novel therapies that may include Brk inhibitors.

Discussion

Although an improved understanding of Brk signaling in breast cancer is emerging (5, 7–9), knowledge of the mechanisms by which Brk is aberrantly expressed in the majority of breast cancers remains incomplete. We have identified cellular microenvironmental stressors, including hypoxia, to be major cues that result in increased Brk expression in both normal and neoplastic mammary epithelial cells. We have additionally shown through ChIP assays and gene expression analysis that this mechanism of Brk transcriptional regulation is HIF-dependent. This finding is particularly relevant to breast cancer subtypes that exhibit high constitutive expression of HIFα subunits, specifically, TNBCs (Fig. 1) and inflammatory breast cancers (19, 21). Importantly, forced expression of Brk in cells lacking both HIF-1α and HIF-2α increased xenograft growth in vivo and promoted lymph node metastasis, suggesting that Brk is a key mediator of the hypoxia-induced metastasis program. Furthermore, using a mouse model of Brk overexpression (WAP-Brk × METMat knockin), we showed that activated Brk signaling drives mammary tumor initiation and rapid tumor growth in vivo. Tumor growth is further accelerated in the presence of activated endogenous Sik (the mouse homolog of Brk, which is not expressed in the normal mammary gland). Sik expression is tightly linked to rapid tumor onset and shortened survival, regardless of Brk transgene genotype. The impact of activated Brk signaling on metastatic progression is of particular interest, as 20% of the Brk × METMat mice developed distant metastases, compared with only 12% of the METMat mice. Clearly, high Brk expression in primary human breast tumors is significantly correlated with decreased overall survival as well as decreased metastasis-free survival (Fig. 7E and F). Further studies, designed specifically to assess differences in metastases [i.e., following removal of primary tumors and/or...
using larger cohorts (43)], are needed to better define the details of how Brk signaling contributes to the stepwise process of metastasis.

Our data suggest that Brk can be upregulated during hypoxia by either HIF-1 or HIF-2 (Fig. 5). Numerous studies have determined that HIF-1α and HIF-2α regulate several of the same genes (20, 35, 36), as expected as the core binding sequence (RCGTG) is recognized by both HIF-1 and HIF-2 (37, 44). Indeed, significant levels of both HIF-1α and HIF-2α have been observed to bind to almost all of the HIF-binding

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<th>Genotype</th>
<th>Number of METs</th>
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Note: Organ with distant metastases were sectioned and H&E-stained. Number of animals harboring distant metastases were quantified and are listed.

aOne mouse had multiple organs with distant metastases.
bP = 0.26 (χ² test).
cP = 0.02 (χ² test).
sites identified by ChIP genome wide. However, functional analysis of the role of individual HREs in the regulation of Brk expression by HIFs is outside the scope of this study. Genome-wide studies have also shown that, despite sharing the same response element, each HIFs subunit also differentially regulates a unique core of distinct genes (37). These studies highlight the complexity of HIF gene regulation and the possibility that HIF-1α and HIF-2α may be involved in both cooperative and compensatory gene regulation that is highly context-dependent (20), and underscore the need for further study. Molecular compensation (i.e., of one HIF for another) is an important consideration for targeting HIF-specific actions in the clinic.

Solid tumors experience widespread cell stress that acts as a form of selection pressure. Our previous studies have elucidated downstream mediators of Brk signaling that include stress-activated protein kinases (p38 mitogen-activated protein kinase (MAPK) and extracellular signal–regulated kinase 5 (ERK5)) and their substrates MEK2 and Sam68 (7–9). Intriguingly, in hTERT-RPE1 cells, HIF-1 transcriptional activity was enhanced by ERK5, and numerous hypoxia-regulated and HIF-1α target genes were specifically regulated by ERK5 (45). These results suggest that HGF, Met, Brk, ERK5, and HIF-1α may function together as a "feed-forward" autocrine signaling loop in response to hypoxia, ultimately resulting in high levels of Brk expression and adaptation to cell stress that enables cancer cells to migrate away from hypoxic regions to more hospitable microenvironments at distant sites. Related to this process is the epithelial-to-mesenchymal transition (EMT), a quintessential step that precedes carcinoma cell metastasis (46). HIF-1α is known to mediate EMT in breast tumors (25) and knockdown of Brk in MCF7 breast cancer cells partially reverses EMT, as measured by a decrease in mesenchymal markers (fibronectin and N-cadherin) and an increase in epithelial markers (E-cadherin and β-catenin; ref. 47). Taken together, these studies suggest that increased Brk expression allows breast cancer cells to successfully migrate, in part via modulation of EMT-associated molecules. Importantly, Brk may act as a convergence point downstream of diverse growth factor receptors that are frequently overexpressed in breast cancer. Targeting Brk (in addition to MET and/or HIF-dependent signaling) as part of combination breast cancer treatment strategies may more effectively subvert HIF-driven processes that contribute to aggressive tumor progression.

Disclosure of Potential Conflicts of Interest

T.N. Seagroves is a consultant/advisory board member of the American Cancer Society. No potential conflicts of interest were disclosed by the other authors.

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References


Correction: Breast Tumor Kinase (Brk/PTK6) Is a Mediator of Hypoxia-Associated Breast Cancer Progression

In this article (Cancer Res 2013;73:5810–20), which was published in the September 15, 2013, issue of Cancer Research (1), the URL on page 5812 is incorrect; the sentence should read as follows:

Survival analysis was conducted using the van’t Veer microarray dataset downloaded from Rosetta Inpharmatics (42).

The online version of the article has been corrected and no longer matches the print.

Reference


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