Breast Tumor Kinase (Brk/PTK6) Is Induced by HIF, Glucocorticoid Receptor, and PELP1-Mediated Stress Signaling in Triple-Negative Breast Cancer

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Abstract

Cancer cells use stress response pathways to sustain their pathogenic behavior. In breast cancer, stress response–associated phenotypes are mediated by the breast tumor kinase, Brk (PTK6), via the hypoxia-inducible factors HIF-1α and HIF-2α. Given that glucocorticoid receptor (GR) is highly expressed in triple-negative breast cancer (TNBC), we investigated cross-talk between stress hormone–driven GR signaling and HIF-regulated physiologic stress. Primary TNBC tumor explants or cell lines treated with the GR ligand dexamethasone exhibited robust induction of Brk mRNA and protein that was HIF1/2a–dependent. HIF and GR coassembled on the BRK promoter in response to either hypoxia or dexamethasone, indicating that Brk is a direct GR/HIF target. Notably, HIF-2α, not HIF-1α, expression was induced by GR signaling, and the important steroid receptor coactivator PELP1 was also found to be induced in a HIF-dependent manner. Mechanistic investigations showed how PELP1 interacted with GR to activate Brk expression and demonstrated that physiologic cell stress, including hypoxia, promoted phosphorylation of GR serine 134, initiating a feed-forward signaling loop that contributed significantly to Brk upregulation. Collectively, our findings linked cellular stress (HIF) and stress hormone (cortisol) signaling in TNBC, identifying the phospho-GR/HIF/PELP1 complex as a potential therapeutic target to limit Brk-driven progression and metastasis in TNBC patients. Cancer Res; 76(6); 1–11. ©2016 AACR.

Introduction

Breast tumor kinase (Brk), also known as protein tyrosine kinase 6 (PTK6), is a soluble tyrosine kinase, distantly related to the c-Src family kinases (1). Although Brk is absent or low in cell line models of normal mammary epithelial cells (2), recent studies show that Brk is elevated but activated (i.e., phosphorylated) and membrane receptor in cancer relative to normal tissues (3). Although Brk undergoes modest to high-level gene amplification in breast tumors (cBioPortal), Brk is most frequently upregulated at the mRNA level (4–6), with highest protein levels in advanced tumor grades (7, 8). Brk is activated downstream of multiple growth factor receptors, including MET, EGF receptor, and ErbB2, and confers aggressive breast cancer phenotypes such as growth factor–induced cell migration, anchorage-independent growth, modulation of EMT markers, metastasis, and resistance to targeted therapies (2, 7, 9–13). Although precocious Brk expression clearly enhances aggressive breast cancer biology (14), a thorough understanding of the mechanisms driving persistent Brk overexpression is lacking.

We demonstrated robust Brk induction following physiologic cell stress stimuli, such as hypoxia, nutrient starvation, and reactive oxygen species (ROS; ref. 2), mediated by the hypoxia-inducible factors (HIF), HIF-1α and HIF-2α, master regulators of responses to physiologic cell stress (15). Although triple-negative breast cancers (TNBC) lack expression of estrogen receptor (ER) and progesterone receptor (PR), glucocorticoid receptor (GR) is highly expressed in 15% to 40% of TNBC tumors (16–18). GRs are members of the nuclear steroid receptor family and bind glucocorticoids (GC). GCs have diverse cell type–specific effects, promoting apoptosis in cells of lymphoid origin and conversely promoting survival in cells of epithelial origin (19, 20). In solid tumors, GR/GCs are emerging as mediators of cell survival and resistance to chemotherapy-induced cell death (21, 22), and GR expression is predictive of decreased survival and increased risk of metastasis in ER+ breast tumors (18).

Herein, we report Brk induction via GR/GC and HIF signaling cross-talk. Our studies demonstrate a novel mechanism of integration of physiologic cell stress (HIF-dependent) and stress hormone (cortisol)–driven pathways, epigenetic signaling events that may drive persistent aggressive tumor cell behavior. Targeting the inducible mediators of tumor progression may lead to increased longevity for breast cancer survivors subjected to chronic therapy during management of metastatic disease.
Materials and Methods
Cell culture
MDA-MB-231 cell lines were obtained in April 2012 from a collaborating laboratory (Dr. Roland Wenger, Institute of Physiology and Zürich Center for Integrative Human Physiology (ZHIP), University of Zürich, Zürich, Switzerland) and cultured, and stable knockdown of HIF1A and HIF2A genes was generated as previously described (2). The MDA-MB-231 cell lines were authenticated on December 8, 2015, by ATCC, and results were compared with the ATCC short-tandem repeat (STR) database. Hs578T and BT20 cell lines were obtained in April 2012 from a collaborating laboratory (Dr. Doug Yee, Masonic Cancer Center, University of Minnesota, Minneapolis, MN) and cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. Cells were maintained in 5% CO2 at 21% O2 (normoxia, ambient air) or at 1% O2 (hypoxia).

Human breast cancer explant experiments
Fresh breast cancer tissues were obtained with informed consent from women undergoing surgery at the Hospitals of the University of Texas Southwestern Medical Center (Dallas, TX; see Supplementary Table S1 for clinicopathologic characteristics). The procedure for establishment of explant followed the previous description (23). The tissues were also either incubated with vehicle (ethanol) alone or dexamethasone (10 μmol/L) and treated 24 hours later with 1% O2 for 6 or 24 hours or 100 μmol/L H2O2 for 1 hour. For experiments requiring hormone treatment, cells were plated and starved (described above) before a 1-hour dexamethasone (1 μmol/L) or H2O2 (100 mmol/L) treatment. Cells were lysed with ELB buffer and lysates were analyzed as described previously.

Immunoblotting
For experiments without hormone treatment, cells were plated and treated 24 hours later with 1% O2 or 6 or 24 hours or 100 μmol/L H2O2 for 1 hour. For experiments requiring hormone treatment, cells were starved for 18 to 24 hours in iMERM with 10% DCC, then cells were treated, if applicable (treatment conditions noted in Figure legends), and whole-cell lysates were isolated as described previously (2) and probed with primary antibodies: Brk (Santa Cruz Biotechnology, sc-1188), GR (Santa Cruz Biotechnology, sc-1003), actin, ERK1/2 (Cell Signaling Technology, 9102L), HIF-1α (Novus Biologicals, NB100-479), HIF-2α (Novus Biologicals, NB100-122), p38 MAPK (Cell Signaling Technology, 9212), phospho-p38 MAPK (Cell Signaling Technology, 4511p), phospho-S134 GR (custom made, Pierce Biotechnology), or PELP1 (Bethyl Laboratories, A300-180A). Representative images of triplicate experiments are shown. Densitometry was determined via ImageJ analysis and normalized to the loading control.

qRT-PCR
qRT-PCR assays were conducted as described previously (2), with MDA-MB-231 cells cultured in normoxia or hypoxia with or without 1 μmol/L dexamethasone or ethanol vehicle for 1 to 24 hours. Target gene expression was normalized to the expression of internal control genes, TATA-binding protein (TBP), Actin, or 18S.

Chromatin immunoprecipitation assays
Chromatin immunoprecipitation (ChIP) assays were conducted as described previously (2), with MDA-MB-231 cells treated with 1 μmol/L dexamethasone or ethanol at either normoxia or hypoxia for 1 hour. ChIP–ReChIP assays were performed; first immunoprecipitating with a GR antibody for 4 hours and subsequently immunoprecipitating was a HIF-2α antibody overnight (18 hours).

Coimmunoprecipitation assays
Coimmunoprecipitation (co-IP) assays were performed as described previously (24). Briefly, MDA-MB-231 or HeLa cells were plated and starved (described above) before a 1-hour dexamethasone (1 μmol/L) or H2O2 (100 mmol/L) treatment. Cells were lysed with ELB buffer and lysates were analyzed as described previously.

Soft agar assays
Soft agar experiments were performed as described previously (25), and results presented are representative of three experimental repeats. Treatment conditions included 10 nmol/L doxorubicin, 1 μmol/L dexamethasone, or both.

Gene expression analysis
Results are represented as means ± SEM. Statistical significance for qRT-PCR and ChIP-qPCR assays was determined via unpaired Student t tests. PELP1 expression was explored among The Cancer Genome Atlas (TCGA) breast tumor samples stratified by clinical IHC triple-negative status (88 TNBC, 434 non-TNBC; ref. 26). The published TCGA median-centered expression data were down-loaded and quantile normalized. PELP1 expression levels were plotted along with their mean ± 95% CI. Welch two-sample t test was performed between the groups.

Results
Glucocorticoids induce Brk in TNBC
In addition to our finding of Brk upregulation via cellular stressors that input to HIF stabilization (2), we sought to investigate Brk regulation by another primary stress-sensing axis, GR/GC signaling. TNBC tumor samples were obtained immediately following surgical resection (Supplementary Table S1). Uniform sets of fresh tumor fragments were maintained on gelatin sponges suspended in media containing vehicle or dexamethasone, a synthetic GC. Notably, 5 of 7 (71%) TNBC tumors showed robust induction of Brk mRNA [and protein] following dexamethasone treatment (24 hours) relative to vehicle-treated internal (i.e., same tumor) controls (Fig. 1A). Brk mRNA expression remained unchanged following dexamethasone treatment in one tumor explant, whereas another showed decreased Brk mRNA expression (Fig. 1A). Brk protein and mRNA induction relative to vehicle control are shown for two representative explants (Fig. 1B). Interestingly, GR protein was also induced in 3 of 4 explants following dexamethasone treatment; two representative explants are shown (Fig. 1B).

To investigate the mechanism of Brk induction by GC signaling, we utilized cell line models of TNBC. MDA-MB-231 cells, which express GR but lack ER or PR, were treated with increasing doses of dexamethasone for 24 hours. Brk mRNA was significantly increased in response to 1 μmol/L dexamethasone treatment relative to vehicle (Fig. 1C). Similarly, Brk mRNA was significantly increased in response to dexamethasone treatment in Hs578T and BT-20 TNBC cell lines. Brk protein was also dose dependently induced in response to dexamethasone and over a time course that peaked at 24 to 30 hours (Supplementary Fig. S1). Our remaining dexamethasone studies were performed using 1 μmol/L dexamethasone, a physiologically relevant dose (27, 28) that is standard in the GR field (29). Together, these data
indicate that GR/GC signaling induces Brk expression in primary human TNBC tumors and cell lines.

GR requires HIFs to induce Brk

To determine whether HIFs are required for GR/GC–induced Brk expression, we utilized MDA-MB-231 cells expressing a nontargeting control shRNA (shControl) or shRNAs specific to both HIF-1α and HIF-2α, resulting in a double knockdown (HIF1/2-shRNA) of HIF-1α and HIF-2α. MDA-MB-231 shControl and HIF1/2-shRNA cells were cultured in normoxia or hypoxia and treated with vehicle or dexamethasone for 24 hours, and Brk mRNA levels were analyzed by qRT-PCR after normalization to actin. TBP, or 18S. *, statistical significance (P < 0.05; an unpaired Student t test); **, statistical significance (P < 0.01; an unpaired Student t test). These data suggest that ligand-activated GR requires HIF-1α and/or HIF-2α to induce robust Brk mRNA expression.

The Brk promoter contains a glucocorticoid response element (GRE) present 852 base pairs upstream of the transcriptional start site, near a known hypoxia response element (HRE), to which we previously demonstrated recruitment of both HIF-1α and HIF-2α during hypoxia (2). ChIP assays showed robust recruitment of GR to the GRE in the Brk promoter following dexamethasone treatment in either cell line model relative to vehicle treatment or IgG-negative controls (Fig. 2C). These data suggest Brk is a direct GR target gene. Ligand-activated GR is also recruited to the BRK promoter in dexamethasone-treated HIF1/2-shRNA cells, although Brk mRNA is not induced, suggesting that HIFs are required for GR/GC coactivation of transcription or other downstream event(s) subsequent to GR recruitment to this region of chromatin.

HIF-2α is a novel GR target gene in TNBC

To test the dependence of HIF-induced Brk expression on GR/GC signaling, MDA-MB-231 cells were cultured in hypoxia or normoxia with or without dexamethasone or the GR antagonist, RU486. As described above (Fig. 1), Brk mRNA was significantly induced in response to dexamethasone treatment, but blocked by the GR antagonist RU486 as expected (Fig. 3A). Brk mRNA was significantly increased in response to hypoxia alone, relative to normoxia, and was further induced upon dexamethasone treatment during hypoxia. Interestingly, when MDA-MB-231 cells were treated with hypoxia, dexamethasone, and RU486 simultaneously, Brk mRNA expression returned to levels seen in hypoxic conditions but were not further reduced to the basal expression levels observed during normoxia. Thus, while GR/GC–induced Brk expression requires HIFs (Fig. 2A), HIFs induce Brk during hypoxia independently of ligand-activated GR (Fig. 3A).

Although the expression of HIF-1α/2α is canonically regulated by oxygen tension through pro teaseal degradation, cancers frequently utilize alternative methods to inappropriately stabilize HIFs (30, 31). We speculated that GR may regulate HIFs in TNBC cells as a means of "presetting" the components necessary for...
rapid upregulation of select target genes, including Brk. To test this, MDA-MB-231 cells were cultured with or without dexamethasone and hypoxia and as expected, HIF-1α and HIF-2α protein levels were substantially induced in hypoxia relative to normoxia. Surprisingly, HIF-2α, but not HIF-1α, protein levels were increased in response to dexamethasone relative to vehicle controls. Alone, dexamethasone or hypoxia significantly induced HIF2A mRNA expression relative to vehicle (Fig. 3B), and combination treatment resulted in additive induction. Similar results were observed in Hs578T TNBC cells (Fig. 3C). To determine whether HIF-2α was a direct GR target gene, we performed ChIP assays to a GRE present in the HIF2A (EPAS1) promoter in MDA-MB-231 cells following dexamethasone and hypoxia treatment and observed robust recruitment of GR in response to dexamethasone treatment compared with vehicle (Fig. 3D). Interestingly, ligand-independent recruitment of GR was observed during hypoxia relative to normoxia and may account for the modest, but significant, increase in HIF2A mRNA observed during hypoxia (Fig. 3B). These data indicate that GR directly binds the EPAS1 promoter prior to induction of HIF2A mRNA in both ligand-dependent and -independent conditions.

HIF-1α/2α are recruited to multiple active regions of the BRK promoter in response to physiologic cell stress stimuli (2). To determine whether GR and HIF-2α were present in the same transcriptional complexes at the BRK promoter in response to hypoxic cell stress and stress hormone exposure, we performed ChIP-REChIP assays. Following first-round immunoprecipitation (IP) with an antibody specific to GR, and subsequent IP with an antibody specific for HIF-2α, we detected strong concomitant recruitment of these transcription factors to the BRK promoter following combined dexamethasone and hypoxia treatment and relative to vehicle controls (Fig. 3E). Thus, GR and HIF-2α interact in the same transcriptional complexes at the BRK promoter following dexamethasone and during hypoxia.

PELP1 interacts with GR to induce Brk expression

Common signaling pathways may exist across diverse hormone-driven cancers. We thus considered common SR coactivators in our models of TNBC. Proline glutamate and leucine-rich protein 1 (PELP1/MNAR) is a known SR coactivator, with important functions in prostate and breast tumor biology and progression (32). PELP1 mRNA expression is significantly higher in TNBC tumors compared with non-TNBC breast tumors (Fig. 4A). We thus evaluated PELP1 protein expression in our ex vivo tumor explant models (as in Fig. 1). Tumor samples (patient #5) were treated with increasing doses of dexamethasone, and PELP1 protein was highly induced following 10 μmol/L dexamethasone treatment relative to vehicle (Fig. 4B). Notably, in the same tumor, Brk and GR protein levels were also robustly induced following dexamethasone treatment. In total, PELP1 was clearly dexamethasone-induced in 4 of 7 tumor explants and tracked with GR induction. Conversely, we observed no increase in PELP1 mRNA or protein following dexamethasone treatment of TNBC cell line models, perhaps due to already high basal PELP1 expression. Alternatively, tumor stromal components may be required for stable dexamethasone-induced PELP1 upregulation (Fig. 4B).

Patient-derived xenograft (PDX) tumors of TNBC had significant enrichment for HIF-1α and HIF-2α relative to similarly propagated luminal tumors (2). To investigate the impact of hypoxia and HIFs on the expression of PELP1 in TNBC cells, MDA-MB-231 shControl or HIF1/2-shRNA cells were cultured in normoxia or hypoxia. Interestingly, PELP1 mRNA was significantly induced in MDA-MB-231 shControl, but not HIF1/2-shRNA (HIF-null) cells cultured in hypoxia relative to normoxia (Fig. 4C). In addition, basal levels of PELP1 mRNA and protein were significantly reduced in HIF1/2-shRNA cells relative to shControl cells (Fig. 4C, inset). ChIP assays demonstrated robust HIF-2α recruitment to an HRE-containing region of the PELP1 promoter in MDA-MB-231 cells in response to hypoxia relative to normoxia (Fig. 4D). Conversely, recruitment of HIF-1α to this region was not consistently detected in hypoxia. Taken together, these data suggest that PELP1 is a HIF-2α target gene in TNBC cells.

We first demonstrated ER/PR/PELP1 signaling and transcriptional complexes in luminal breast cancer models (33). However,
no studies have defined a role for GR in PELP1-containing transcriptional complexes in breast cancer cells. Co-IP experiments performed in MDA-MB-231 cells treated with or without dexamethasone revealed basal GR interaction with PELP1 that increased upon dexamethasone treatment relative to vehicle controls (Fig. 5A). Similar results were observed in Hs578T TNBC cells (Fig. 5B). ChIP assays in MDA-MB-231 cells treated with or without dexamethasone showed robust PELP1 recruitment to the GRE located in the BRK promoter following dexamethasone treatment compared with vehicle (Fig. 5C). Together, these data indicate that GR and PELP1 interact in whole-cell lysates and are recruited to the same location in the BRK promoter in response to dexamethasone treatment. To determine whether PELP1 is required for dexamethasone-induced recruitment of both GR and PELP1 to the BRK promoter in cells subjected to D2 pretreatment relative to dexamethasone treatment alone (Fig. 5E and 5F). Thus, PELP1 is a key cofactor for GR/GC induction of BRK expression in TNBC cells.

Phosphorylation of GR S134 is important for BRK induction

Ligand-independent phosphorylation of GR occurs at serine 134 (S134) via p38 MAPK in response to physiologic cell stress stimuli in U2OS osteosarcoma cells (34). We hypothesized that phosphorylation of GR-S134 provides a mechanistic link between HIF- and hormone- (GC) mediated cell stress–induced inputs to BRK upregulation in TNBC cells. MDA-MB-231 cells were treated with increasing doses of H2O2, and phosphorylation of GR-S134 was visualized by Western blotting using phospho-S134 antibodies. H2O2 treatment resulted in enhanced phosphorylation of GR-S134 (Fig. 6A). Notably, we observed constitutive, basal phosphorylation of GR-S134, as previously observed in U2OS cells (34), as well as activation of p38 MAPK. To determine whether GR-S134 is phosphorylated in response to hypoxic cell stress, MDA-MB-231 cells and HeLa cells were cultured in hypoxia or with H2O2, as a positive control. Phosphorylation of GR-S134 increased at 6 and 24 hours of hypoxia relative to normoxia in both cell lines (Fig. 6B), whereas total GR protein levels remained unchanged. We next assessed the phosphorylation of GR-S134 in two TNBC PDX cell lines, HCl-2 and HCl-10, previously shown to have high levels of HIF-1α, HIF-2α, and BRK protein expression (2). Hypoxia induced robust GR-S134 phosphorylation in both HCl-2 and HCl-10 TNBC models (Fig. 6C).

Phospho-GR/HIF/PELP1 Complexes Induce Brk/PTK6 in TNBC

Figure 3.

GR regulates HIF-2α expression, and GR/HIF-2α are corecruited to the BRK promoter. A, MDA-MB-231 cells cultured in normoxia or hypoxia for 24 hours with vehicle, 1 μM dexamethasone (Dex), 1 μM RU486, or both agents, and mRNA levels were assessed by qRT-PCR after normalization to TBP levels. Asterisks indicate statistical significance from all other treatment groups in either normoxia or hypoxia. B, MDA-MB-231 cells were treated for 24 hours with vehicle, 1 μM dexamethasone, hypoxia, or both agents and subjected to Western blot analysis for HIF-1α, HIF-2α, or ERK1/2 (loading control). Brk mRNA levels were analyzed by qRT-PCR after normalization to TBP levels. D = H2O2, dexamethasone + hypoxia. C, Hs578T cells treated with vehicle or 1 μM dexamethasone for 24 hours and mRNA expression was analyzed via qRT-PCR following normalization to TBP expression. D, MDA-MB-231 cells were cultured at normoxia or hypoxia and treated with vehicle or 1 μM dexamethasone for 1 hour, and ChIP assays were performed. Negative isotype-matched IgG controls were conducted on hypoxia- and dexamethasone-treated MDA-MB-231. E, MDA-MB-231 cells were treated with vehicle or 1 μM dexamethasone and hypoxia for 1 hour. ChIP-Re-ChIP assays were performed with initial immunoprecipitation with GR antibody and subsequently immunoprecipitation with HIF-2α antibody. No secondary antibody was included in control samples to demonstrate specificity. Representative examples from triplicate experiments are shown. (*, P < 0.05; **, P < 0.01; ****, P < 0.001; unpaired Student t test).
expressed higher levels of both total and phospho-S134 GR and also contained higher levels of Brk relative to patients #9 and #11. These data support our in vitro findings that Brk expression tracks with phospho-S134 GR in TNBC.

To test the requirement for GR-S134 phosphorylation in GR/PELP1 interaction in TNBC cells, co-IP assays were performed with MDA-MB-231 cells. Interestingly, increased levels of GR were seen in PELP1 immunoprecipitates following H2O2 treatment (i.e., to induce phosphorylation of GR-S134) relative to vehicle (Fig. 6E). To definitively assess the requirement of this phosphorylation event, co-IP assays were conducted in MDA-MB-231 cells stably expressing either flag-tagged wild-type (WT) GR (WT flag-GR) or a mutant GR, in which S134 was mutated to a nonphosphorylated alanine residue (S134A flag-GR). We observed decreased PELP1 in S134A flag-GR immunoprecipitates relative to levels present in WT flag-GR immunoprecipitates, both basally and in response to dexamethasone treatment (Fig. 6F). These data suggest that GR phosphorylation at S134 occurs during hypoxic stress and facilitates or stabilizes GR/PELP1 association.

Highest levels of phosphorylation of GR-S134 were observed following exposure to H2O2 (i.e., during generation of ROS) in MDA-MB-231 cells (Fig. 6). To implicate phosphorylation of GR-S134 on dexamethasone-induced GR recruitment to the BRK promoter, MDA-MB-231 cells were treated with or without dexamethasone, H2O2, or both agents, and GR recruitment was assessed via ChIP assay. As previously seen, GR was recruited to the BRK promoter following dexamethasone treatment relative to vehicle. We also detected a slight increase in GR recruitment to the BRK promoter in the presence of H2O2 alone. Notably, combined dexamethasone and H2O2 treatment further enhanced recruitment of GR to the BRK promoter relative to either dexamethasone or H2O2 treatment alone (Fig. 7A). We then tested the requirement for p38 MAPK activity in dexamethasone-induced recruitment of GR to the BRK promoter. MDA-MB-231 cells were pretreated with SB203580, a p38 MAPK inhibitor, which effectively blocks phosphorylation of GR-S134 (Fig. 7B; inset), followed by dexamethasone or vehicle treatment. SB203580 pre-treatment resulted in diminished basal recruitment of unliganded GR to the BRK promoter relative to vehicle control (Fig. 7B). Interestingly, combined treatment with both SB203580 and dexamethasone substantially decreased GR recruitment to the BRK promoter relative to dexamethasone alone. These data suggest that inhibition of GR-S134 phosphorylation hinders the ability of GR to associate with a GRE-containing region of the BRK promoter. Regulation of two classic GR target genes, DUSP1 and GILZ, remained unaltered in similar conditions (Supplementary Fig. S2C–F); both were robustly dexamethasone-induced in HIF1/2-shRNA cells relative to vehicle controls (Supplementary Fig. S2A and B), demonstrating the specificity of phospho-GR-S134 target gene selection.

Finally, to definitively test the requirement of GR-S134 phosphorylation for recruitment of GR to the BRK promoter, we performed ChIP assays with MDA-MB-231 cells expressing either WT flag-GR or S134A flag-GR. As expected, WT flag-GR was recruited to the BRK promoter following dexamethasone treatment relative to vehicle controls. However, the association of S134A flag-GR with the BRK promoter was basally reduced and was not significantly increased in response to dexamethasone.
relative to vehicle controls (Fig. 7C). These data suggest that phosphorylation of GR-S134 is required for recruitment of ligand-bound GR to the BRK promoter. Our data collectively suggest a mechanism through which PELP1, GR, and HIF-2α cooperatively induce Brk expression following physiologic (HIF) and hormone (GC) stress signaling. Indeed, ChIP assays confirmed that all three molecules are simultaneously recruited to the same region of the BRK promoter in response to stress stimuli in MDA-MB-231 treated with dexamethasone and H2O2. (Fig. 7D). To link GR/GC–induced TNBC cell survival to Brk expression, we performed soft agar assays with MDA-MB-231 cells expressing shControl, HIF1/2-shRNA (Brk-null), or HIF1/2-shRNA+Brk (in which Brk expression is restored), grown in the presence of doxorubicin with or without dexamethasone. MDA-MB-231 shControl cells treated with dexamethasone exhibited significantly more colonies per field relative to doxorubicin treatment alone (Fig. 7E). Brk-null HIF1/2-shRNA cells exhibited decreased ability to form colonies in the presence of doxorubicin and complete loss of dexamethasone-mediated cell survival. Notably, HIF1/2-shRNA+Brk cells exhibited increased colony formation with doxorubicin treatment relative to Brk-null models, and importantly, the protective effect of dexamethasone treatment was restored. These data suggest that HIFs are required for GR/GC–induced cell survival, but that exogenous Brk expression can bypass this requirement, perhaps in part via reestablishment of feed-forward signaling (i.e., via Brk-induced activation of p38 MAPK) at additional phospho-S134 GR and PELP1 target genes (Fig. 7F).

Discussion

Our data demonstrate a remarkable hormone (GC)-dependent signaling pathway, wherein GR-S134 acts as an additional “stress sensor” of cellular stressors (ROS, hypoxia, etc.) that stabilize HIFs. This phosphorylation event facilitates GR/PELP1 interactions at novel GR/PELP1/HIF target genes typified by Brk, an important mediator of advanced cancer phenotypes and tumor progression. This mechanism of integration of HIF-dependent cell stress signaling pathways with GR/GC signaling suggests that these pathways may overlap more than previously thought. Notably, many HIF responsive genes have functions relevant to cancer biology, such as glucose metabolism, angiogenesis, and cell migration (35). HIF-α overexpression in breast cancer is predictive of relapse and higher risk of metastasis, and high levels of HIF-1α are specifically associated with TNBC (36, 37). Similarly, HIF-2α is emerging as an important mediator of cancer metastasis (38). Targeting PELP1, HIFs (including HIF-2α), and/or blocking GR-S134 phosphorylation via inhibition of upstream p38 MAPK signaling may provide a means of “redirecting” GR away from genes that promote prosurvival and tumor progression during cancer chemotherapy while preserving the desired protective (i.e., to inflammation) effects of therapeutic corticosteroids.

Remarkably, PELP1 expression is HIF-dependent (Fig. 4C). PELP1 is primarily associated with ER or androgen receptor (AR) transactivation but can also transrepress and transactivate GR in a cell type–specific manner (39). Our data support a role for PELP1 as an important GR coactivator. Like GR, PELP1 mRNA expression is significantly increased in TNBC compared with non-TNBC (Fig. 4A). PELP1 expression is dysregulated in multiple cancer types, including 60% to 80% of breast tumors (40, 41). Notably, high PELP1 expression in breast tumors is associated with increased tumor grade, cell proliferation, metastasis, and decreased disease-free survival, as well as the appearance of basal cytokeratin markers (40–43). Patients with PELP1/Ki-67 double high tumors experienced shorter disease-free survival and overall survival (44). Expression of PELP1 is inversely associated with expression of ER, PR, or AR (41). Ours
is the first study to link PELP1 expression and function to GR. Consistent with the concept of GR is a key mediator of increased TNBC cell survival (Fig. 7E; refs. 18, 29, 45), PELP1 knockdown in mutant p53 TNBC cells enhanced chemotherapeutic-induced cell death, in part via modulation of gain-of-function mutant p53 activity (46). PELP1 inhibitors are currently in development, primarily for prostate cancer and ER/PR-positive luminal breast tumors (23). Our studies suggest that PELP1 inhibitors may have utility in treating aggressive TNBC, especially in patients whose tumors exhibit PELP1/Ki-67 double high expression (44). Like Brk (47), GR is a mediator of prosurvival in breast cancer. TNBC cells treated with combination chemotherapy and dexamethasone, in vitro and in vivo, undergo significantly decreased cell death relative to chemotherapy alone (29). Pang and colleagues identified multiple GR target genes that mediate this prosurvival effect, such as SGK-1 and dual-specificity serine phosphatase 1 (DUSP1; refs. 29, 45). Relevant to these findings, increased phosphorylation of GR at S134 resulted in association of GR with the adaptor protein 14-3-3zeta and ultimately globally changed GR target genes in response to dexamethasone treatment of osteosarcoma cell models (34). Notably, as with Brk, GR, PELP1, and HIFs, expression of 14-3-3zeta is also very high in TNBC (i.e., as determined via TCGA analysis), suggesting that these molecules cooperate to alter gene expression and cell fate in TNBC. Our current study identifies phospho-S134 GR as a critical regulator of novel GR/PELP1/HIF complexes. We suspect that phospho-S134-GR/PELP1/HIF signaling complexes are markers of aggressive tumors and drive a new gene program to promote tumor cell prosurvival and progression to metastasis. As Brk is known to promote these aggressive phenotypes, it is likely a key downstream effector of GR/HIF-dependent stress signaling in TNBC.

Grk may be representative of a larger gene program that is jointly regulated by HIFs, phospho-GR, 14-3-3zeta, and PELP1. Going forward, it will be important to identify risks associated with GC-based therapies. Breast cancer patients typically receive high-dose GC treatment prior to chemotherapy to alleviate adverse side effects. Furthermore, organ transplant patients chronically treated with GCs to achieve immunosuppression (48) experience greatly increased rates of metastatic melanoma (49), an aggressive cancer that is typically Brk+ (50). Thus, a detailed understanding GR/GC signaling in cancer biology and its impact on aggressive tumor phenotypes is urgently needed. Our studies demonstrate a novel feed-forward loop, in which components of the stress pathways (p38 MAPK, Brk, HIFs, GR, and PELP1)

Figure 6.

GR S134 is phosphorylated in hypoxia and required for GR/PELP1 interaction. A, MDA-MB-231 cells were treated with vehicle or increasing doses of H2O2 for 1 hour, and Western blot analysis was performed with antibodies to phospho-S134, total GR, phospho-p38 MAPK, or total p38 MAPK (loading control). B, MDA-MB-231 or HeLa cells were cultured in normoxia or hypoxia for 6 or 24 hours or with 100 μmol/L H2O2 (positive control) for 1 hour and subjected to Western blot analysis with antibodies to phospho-S134, total GR, or ERK1/2 (loading control). C, cell lines established from PDXs were cultured ex vivo for 24 hours at normoxia or hypoxia, and lysates were subjected to Western blot analysis for phospho-S134, total GR, or ERK1/2 (loading control). D, whole-cell lysates from five primary patient samples of TNBC were subjected to Western blot analysis with antibodies for total GR, phospho-S134, Brk, or β-actin (loading control). E, MDAMB-231 cells were pretreated for 30 minutes with 100 μmol/L H2O2 followed by 1-hour vehicle or 1 μmol/L dexamethasone (Dex) treatment and subjected to immunoprecipitation (IP) with PELP1 or rabbit IgG (control) antibodies. Immunoprecipitation lysates or input lysates were analyzed by Western blotting for PELP1 and GR. F, MDA-MB-231 cells stably transfected with Flag-WT GR or Flag-S134A GR constructs were treated for 1 hour with 1 μmol/L dexamethasone or vehicle and subjected to immunoprecipitation with Flag antiserum or mouse IgG (control) antibody. Immunoprecipitation lysates and input lysates were assessed by Western blotting using Flag or PELP1 antiserum. Relative levels of PELP1 expression, via densitometry, are shown.
regulate the expression and activity of other members of the pathway (i.e. Brk activates p38 MAPK, and GR induces HIF-2α, while HIFs induce PELP1, which binds to phospho-GR), to ultimately potentiate stress signaling, persistently augment pathway activity, and drive gene expression (i.e., epigenetic events typified by Brk induction) required for aggressive tumor biology (Fig. 7F).

Disclosure of Potential Conflicts of Interest
G.V. Raj has ownership interest (including patents) in Peptidomimetics. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: T.M. Regan Anderson, G.V. Raj, C.A. Lange
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Figure 7.
The phospho-GR/HIF/PELP1 complex is recruited to the BRK promoter in response to stress. A and B, MDA-MB-231 cells were pretreated with 100 μmol/L H2O2 (A) or 10 μmol/L SB203580 or DMSO (B) for 30 minutes followed by 1-hour vehicle or 1 μmol/L dexamethasone (Dex) treatment, or ChIP assays were performed with GR antisera or negative isotype-matched control antibodies and qRT-PCR was performed. Inset, Western blot analysis of MDA-MB-231 cells pretreated with 10 μmol/L SB203580 for 30 minutes followed by 100 μmol/L H2O2 treatment for 1 hour and probed with antibodies for phospho-S134, GR, or ERK1/2 (loading control). C, MDA-MB-231 cells stably expressing flag-WT GR or Flag-SA GR were treated with 1 μmol/L dexamethasone for 1 hour, and ChIP assays with flag antibodies or negative isotype-matched controls were performed. Isolated DNA was assessed by qRT-PCR. D, MDA-MB-231 cells were treated for 1 hour with 1 μmol/L dexamethasone and 100 μmol/L H2O2. ChIP assays were then performed with antisera for PELP1, GR, and HIF-2α or negative isotype-matched control. qRT-PCR was performed on isolated DNA. A representative experiment is shown from triplicate experiments. E, soft agar colony formation assays with MDA-MB-231 shControl, HIF1/2-shRNA, and HIF1/2-shRNA+Brk cells grown in 10 nm doxorubicin with 1 μmol/L dexamethasone or vehicle for 18 days. F, model detailing feed-forward phospho-GR/HIF/PELP1 signaling loop. Stress stimuli stabilize HIFs and activate p38 MAPK; p38 phosphorylates GR on S134. Cortisol-bound GR induces HIF2A expression and HIFs induce PELP1. Phospho-S134 GR binds to PELP1, leading to formation of phospho-GR/HIF2/PELP1 complexes and induction of Brk mRNA expression.
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