Acquisition of the Metastatic Phenotype Is Accompanied by H$_2$O$_2$-Dependent Activation of the p130Cas Signaling Complex

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Abstract
Reactive oxygen species (ROS) have emerged as cellular signaling molecules and are implicated in metastatic disease by their ability to drive invasion and migration. Here, we define the signaling adaptor protein p130Cas (Crk-associated substrate) as a key redox-responsive molecular trigger that is engaged in highly invasive metastatic bladder tumor cell lines. Endogenous shifts in steady-state hydrogen peroxide (H$_2$O$_2$) that accompany the metastatic phenotype increase p130Cas phosphorylation, membrane recruitment and association with the scaffolding protein Crk, and subsequent Rac1 activation and actin reorganization. Both enzymatic and nonenzymatic scavenging of H$_2$O$_2$ abrogates p130Cas-dependent signaling and the migratory and invasive activity of the metastatic bladder tumor cells. Disruption of p130Cas attenuates both invasion and migration of the metastatic variant (253J-BV). 253J-BV cells displayed an increase in global thiol oxidation and a concomitant decrease in total phosphatase activity, common target proteins of active-site cysteine oxidation. The dependence of phosphatases on regulation of p130Cas was highlighted when depletion of PTPN12 enhanced p130cas phosphorylation and the migratory behavior of a noninvasive parental bladder tumor control (253J). These data show that the metastatic phenotype is accompanied by increases in steady-state H$_2$O$_2$ production that drive promigratory signaling and suggest that antioxidant-based therapeutics may prove useful in limiting bladder tumor invasiveness. Mol Cancer Res; 11(3); 303–12. ©2013 AACR.

Introduction
The ability of a tumor cell to invade and migrate through the basement membrane and extracellular matrix (ECM) is a vital first step in tumor metastasis. Tumor cell invasion is accompanied by protease-driven ECM degradation and reorganization of cellular signaling components at the leading and lagging edges of the cell, ultimately leading to restructuring of the actin cytoskeleton and migration. An important signaling cascade involved in this process is the focal adhesion kinase (FAK) pathway. Initiation of this cascade involves phosphorylation and engagement of the kinases FAK and Src, leading to recruitment of adaptor proteins such as p130cas (Crk-associated substrate) and Crk to the focal contacts at the leading edge. This complex engages DOC180, a guanine nucleotide exchange factor, to activate Rac-1 and consequential actin rearrangement.

Reactive oxygen species (ROS) are known DNA damaging agents involved in carcinogenesis. However, at sublethal levels, ROS are implicated in regulating a multitude of cellular signaling cascades, primarily due to their ability to reversibly oxidize thiolate anions of cysteine residues (1, 2). Endogenous ROS are often elevated in cancer cells and this is accompanied by alterations in the cells’ antioxidant-scavenging potential (3–5). Thus, cancer cells have uniquely acquired the ability to survive and even thrive despite this chronic, sublethal oxidant challenge.

Intracellular ROS production has been linked to metastatic disease progression (6). The loss of mitochondrial genome integrity is accompanied by an increase in ROS levels that enhance the metastatic potential of tumor cells (7). ROS of both mitochondrial and nonmitochondrial origin are associated with regulating cellular signaling pathways, which are implicated in metastatic disease (2, 8). We have shown that enhanced invasion and migration are associated with intrinsic increases in the intracellular hydrogen peroxide (H$_2$O$_2$) milieu and changes in antioxidant expression, using a model of metastatic bladder cancer progression (9, 10). This represents a pathophysiologically relevant model that does not rely on manipulation of mitochondrial genome integrity (7); NADPH oxidase stimulation, following receptor or integrin engagement (6), nor manipulating antioxidant expression (11, 12) to change intracellular redox status. Two-fold picomolar increases in intracellular H$_2$O$_2$ (18 $\rightarrow$ 32 pmol/L) in the metastatic
bladder cancer cell variant 253J-BV are associated with enhanced matrix metalloproteinase (MMP)-9 and VEGF expression and increased clonogenicity compared with the related parental cells 253J (9, 10). Here, we set out to test the hypothesis that metastatic cancer cells have uniquely adapted to thrive with an enhanced intracellular ROS milieu and evolved to use oxidation as a novel mechanism to drive promigratory signaling events by H₂O₂-dependent activation of the p130Cas signaling complex.

Materials and Methods

Cell culture, treatments, and transfection

253J and 253J-BV cells were created and cultured as described previously (9, 10). Cells were treated with H₂O₂ (Sigma-Aldrich) in serum-free Dulbecco’s modified Eagle’s medium (DMEM). Cells were pretreated in DMEM +10% FBS with either 500 U/mL bovine catalase (CAT; Sigma-Aldrich) or 2 mmol/L N-acetyl-l-cysteine (NAC; Sigma-Aldrich) for 18 to 24 hours, followed by the same treatments in serum-free media for the duration of the experiments, as indicated. Alternatively, CAT or control β-galactosidase (Lac-Z; MOI 100) were expressed using adenoviral gene delivery (10). CAT activity was determined as described previously (10).

The following siRNA constructs were transfected into 200,000 cells using RNAi Max Lipofectamine reagent (Invitrogen Life Technologies): 200 pmol PTPN12 Silencer Select siRNA (Ambion), 100 pmol p130Cas siRNA (Santa Cruz Biotechnology), or equal molar concentration of Nontargeting siRNA (control #1, Dharmacon-Thermo Scientific).

Wound healing and Matrigel invasion assays

Wound healing and Matrigel invasion assays were essentially carried out as previously described (12). For Matrigel transwell (BD Biosciences) experiments, 25,000 253J-BV cells were applied to the top of the chamber, with indicated treatments, and allowed to invade (24 hours) toward 10% serum DMEM as the chemoattractant. Cells invading to the filter were stained (0.8% NaCl, 50% ethanol, 5% formaldehyde, and 0.2% crystal violet), and images were acquired using a Zeiss Axio Observer.Z1 with an EC Plan-Neofluar 5×/0.15 M27 objective and Axiocam MR3. No appreciable invasion through Matrigel could be observed with 253J cells with the same assay conditions.

Antibodies, immunoblotting, and immunoprecipitation

The following antibodies were used in the study: Crk, p130Cas, anti-fluorescein isothiocyanate (FITC; BD Biosciences), phospho-p130Cas Y165, Fak Y397, Src, Src Y416 (Cell Signaling), PTP-PEST, EGF-R (Santa Cruz Biotechnology), PTPN12, CAT, PY20 phospho tyrosine (Abcam), gliceraldehyde-3-phosphate dehydrogenase (GAPDH; Ambion Life Technologies). Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer [150 mmol/L NaCl, 50 mmol/L Tris pH 7.5, 1% NP-40, 0.5% deoxycholate, protease inhibitor cocktail (Roche), 1 mmol/L sodium orthovanadate]. Cleared lysates were quantified for protein concentration (BCA, Thermo Scientific) and loaded on either 4% to 12% NuPAGE (Life Technologies) or 10% SDS-PAGE. For immunoprecipitation, protein (1 mg) was incubated with anti-Crk antibody (1 μg) and protein G sepharose, and eluded in 2× reducing SDS-PAGE buffer. Following electrophoresis, transfer to nitrocellulose membrane (iBlot, Life Technologies) and blocking, primary antibody incubation was carried out in 5% bovine serum albumin (BSA) TBS-0.1% Tween (1:1,000). Secondary horseradish peroxidase–conjugated mouse or rabbit antibodies (GE Healthcare) at 1:10,000 dilutions were added followed by chemiluminescence detection (Pico & Femto ECL reagents, Thermo Scientific).

Membrane/cytoskeletal fractionation

The membrane/cytoskeletal fractions were obtained using a protocol adapted from Zhao and colleagues (13). Cells were lyzed in cytoskeleton (CSK) buffer [0.5% Triton X-100, 10 mmol/L Tris–HCl (pH 6.8), 50 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L MgCl₂, 1 mmol/L sodium orthovanadate, and protease inhibitors] at 4°C. Following centrifugation (13,600 × g, 30 minutes, 4°C), the supernatant, Triton X soluble cytosolic fraction was removed and analyzed for protein content. The Triton X insoluble pellet (membrane/ cytoskeletal fraction) was washed, resuspended in SDS buffer [1% SDS, 10 mmol/L Tris–HCl (pH 7.5), 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, and protease inhibitors] and boiled. The supernatant Triton X insoluble fraction was cleared from cell debris by centrifugation (13,600 × g, 30 minutes 4°C). Equivalent amounts of Triton X insoluble fraction in reference to soluble fraction protein concentration were electrophoresed, followed by immunoblotting.

Immunofluorescence staining

Cells grown on glass coverslips were fixed with 4% paraformaldehyde/PBS, permeabilized (0.1% Triton X 100/PBS) and blocked (3% FBS/PBS). Incubation with phospho-p130Cas Y165 antibody (1:100) was followed by anti-rabbit Alexa-Fluor 488–conjugated antibody (Life Technologies, 1:1,000). F-actin was stained with Phalloidin Texas-Red (Life Technologies, 1:50) and nuclear DNA with 4',6-diamidino-2-phenylindole (DAPI). Cells were mounted (Prolong Gold Antifade, Life Technologies) and images taken on a Zeiss Axio Observer.Z1 with an EC Plan-Neofluar 63×1.25 NA objective and Axiocam MR3. No appreciable invasion through Matrigel could be observed with 253J cells with the same assay conditions.

Rac1 activation assay

Active GTP-bound Rac1 was precipitated from lysates using the p21-binding domain (PBD) of p21-activated...
protein kinase (PAK1) bound to agarose beads. The Rac1/Cdc42 Activation Assay was purchased from Millipore and carried out as per manufacturer’s instructions.

**Iodoacetamide labeling of disulfides and reduced cysteines**

In situ reverse 5-iodoacetamide fluorescein (5-IAF) labeling was adapted from Yang and colleagues (14). Following H$_2$O$_2$ treatment, cells were fixed in methanol and permeabilized (Triton X 100). Free/reduced cysteines were blocked with 200 mmol/L iodoacetic acid (IAA; Sigma-Aldrich) in 100 mmol/L Tris (pH8.3), 5 mmol/L EDTA (30 minutes, room temperature), with IAA alkylated residues being protected from this reduction step. Re-reduced thiols were subsequently labeled with 1 mmol/L dithiothreitol (DTT), 100 mmol/L Tris (pH8.3), 5 mmol/L EDTA (30 minutes, room temperature) and cells mounted (Prolong). Images were taken as described earlier, background corrected and fluorescence intensity quantified using Fluoview software.

**Protein phosphatase activity assay**

Total phosphatase activity of cellular lysates was assessed using chlorimetric analysis of dephosphorylation of paranitrophenol phosphate (pNPP, Thermo Scientific) according to Streit and colleagues (15).

**Statistical analysis**

All figures are representative of at least 3 replicate experiments. Data are presented as mean ± SEM. The t tests and one-way ANOVA with Tukey multiple comparison posttests were conducted using Prism 5.01 (GraphPad Software).

**Results**

**Intracellular increases in ROS contribute to enhanced migration and invasion of metastatic bladder cancer cells**

The highly metastatic 253J-BV cell line was derived from a poorly metastatic parental human bladder carcinoma cell line, 253J, following 5 successive bladder xenografts (9). We have previously shown that 253J-BV cells display a nearly 2-fold increase in intracellular steady-state H$_2$O$_2$ compared with 253J cells (18 → 32 pmol/L; ref. 10). 253J-BV cells also exhibit an altered antioxidant profile, with increased manganese superoxide dismutase (MnSOD) expression, decreased CAT expression, and a decrease in reduced to oxidized glutathione ratio (GSH/GSSG; ref. 10). We next asked whether the metastatic 253J-BV cell line displays increases in its in vitro migratory and invasive behavior. Using a classical scratch-wound assay to measure basic cell migration parameters, the metastatic 253J-BV variant exhibited enhanced migration in vitro compared with the parental 253J line (Fig. 1A). Similarly, using Matrigel-coated transwell assays to assess invasion, only the 253J-BV cells were able to invade through the Matrigel matrix. Addition of the H$_2$O$_2$-detoxifying enzyme CAT or the antioxidant NAC significantly attenuated the migratory capacity of 253J-BV cells (Fig. 1B). 253J-BV cell invasion was also impaired by CAT and NAC treatments (Fig. 1C). Treatment of both cells with low dose H$_2$O$_2$ (5–50 µmol/L) stimulated migration (Fig. 1D). This low dose H$_2$O$_2$ treatment did not result in cytotoxicity to either cell line. Interestingly, the basal migration rate of 253J cells was not significantly altered by CAT or NAC treatment (Supplementary Fig. S1). These data implicate ROS as participants in regulating the migratory and invasive behavior of the metastatic 253J-BV cells.

**Redox-dependent p130Cas phosphorylation regulates focal adhesion kinase signaling**

Because of the important contribuion of ROS in cellular signaling, we monitored whether shifts in steady-state H$_2$O$_2$ augment prometastatic signaling networks within 253J-BV cells. We first evaluated the phosphorylation state of FAK, as it plays an important role in cancer cell migration and is redox-responsive (16–19). We found that both total FAK and its (Y397) phosphorylation were moderately elevated in 253J-BV cells and this was attenuated by CAT treatment (Fig. 2A). FAK$^{397}$ creates a binding site for Src kinase whose (Y416) phosphorylation state remained constant between the 2 cell lines. Interestingly, total Src levels were diminished in 253J-BV lysates relative to the 253J parental cells, and may reflect a depletion of its cytosolic pools. This finding may suggest that Src$^{416}$ predominates in the metastatic variant, which in turn facilitates FAK-Src signaling. Interestingly, Src phosphorylation remained unchanged following CAT treatment (Fig. 2A).

Active FAK-Src facilitates p130Cas binding and phosphorylation. The adaptor protein p130Cas links FAK-Src to DOC180, enabling this guanine nucleotide exchange factor to activate Rac-1. Phosphorylation of p130Cas (Y165) was robustly enhanced in 253J-BV cells (4.2 ± 0.7-fold compared with 253J) and this increase was attenuated by 68% following treatment with exogenous CAT (Fig. 2A) or by adenoviral-mediated CAT expression (Fig. 2B), indicating that phospho-p130Cas status is H$_2$O$_2$-dependent. Conversely, p130Cas phosphorylation was increased in nonmetastatic 253J cells following treatment with low dose H$_2$O$_2$ (Fig. 2C). The effect of exogenous H$_2$O$_2$ treatment was less evident in 253J-BV cells, presumably due to the high endogenous phospho-status of p130Cas in the metastatic cells.

**Intracellular redox status regulates membrane localization of p130Cas**

Phosphorylation of p130Cas is necessary for its membrane recruitment to focal contacts in migrating cells (20, 21). Cellular fractionation revealed that total and phosphorylated p130Cas$^{165}$ levels were significantly higher in membrane fractions from 253J-BV cells under control conditions compared with the parental line (Fig. 3A and 3B). Membrane association of both p130Cas and p130Cas$^{165}$ were significantly enhanced in nonmetastatic 253J cells following treatment with increasing concentrations of H$_2$O$_2$ (Fig. 3A and B; see Supplementary...
Phosphorylation occurred rapidly and was observed as early as 10 minutes post-H2O2 treatment. Furthermore, 253J-BV cells displayed enhanced p130CasY165 localization to membrane protrusions, as assessed by immunofluorescence. Equivalent analysis of the nonmetastatic 253J cells revealed a distinct uniform cellular distribution of p130CasY165 (Fig. 3C). In addition, 253J-BV cells exhibited differential actin cytoskeletal distribution, with enhanced accumulation of actin fibers at the cell periphery. The localization of p130Cas was also strongly enhanced in filopodia-like protrusions of metastatic cells. In the scratch-wound assay, the frequency of p130CasY165-positive protrusions at the leading edge of 253J-BV cells was quantified and shown to be significantly higher than in 253J cells (Fig. 3D, E, and H). Treatment of 253J cells with 50 μmol/L H2O2 significantly increased the number of positive protrusions for p130CasY165 (Fig. 3D, E, and H). Conversely, CAT treatment drastically reduced p130CasY165-positive protrusions in 253J-BV cells (Fig. 3F, G, and H). These data suggest that the increases in p130CasY165 in 253J-BV cells, relative to 253J cells, are in large part redox-dependent and likely contribute to the redistribution of F-actin in membrane protrusions of metastatic cells.

**Cellular redox state influences p130Cas-mediated downstream signaling**

Following phosphorylation and membrane localization, p130Cas associates with Crk to initiate GTPase recruitment and downstream signaling. Analysis of Crk-associated p130Cas using coimmunoprecipitation revealed a...
marked increase in p130Cas-bound Crk in the metastatic 253J-BV cells that was attenuated by CAT treatment (Fig. 4A). Furthermore, the amount of active GTP-bound Rac-1 was decreased in the 253J-BV cells, following both adenoviral CAT expression and treatment with exogenous CAT (Fig. 4B). These findings indicate that p130Cas association with Crk is under redox-control and likely participates in the robust migratory signaling activity of the metastatic 253J-BV cells. To directly assess the role of p130Cas in migratory signaling, we silenced its expression in both the 253J and 253J-BV cells and observed a significant decrease in the migratory behavior of both cell lines as compared with control siRNA-treated cells (Fig. 4C). The impact of p130Cas suppression on 253J-BV invasion was more pronounced, displaying a near complete inhibition of invasion (Fig. 4D). The nonmetastatic 253J cells display no invasive activity, thus the impact on p130Cas suppression using this assay was not assessed. These findings clearly establish the important contribution of p130Cas to the invasive activity of the metastatic 253J-BV bladder cell line.

**Redox status of metastatic bladder cancer cells influences global protein thiol disulfide formation and protein tyrosine phosphatase inactivation**

ROS play an important role as second messengers in cellular signaling through reversible, oxidative inactivation of regulatory phosphatases (22). Protein tyrosine phosphatases (PTP) contain active site cysteines, which are susceptible to thiol oxidation due to their low pKa. This oxidation can lead to a number of reversible modifications, such as sulfenic acid and disulfide formation. Thus, oxidative phosphatase inactivation is commonly linked to increases in kinase activity. We hypothesized that increases in steady state H2O2 in the 253J-BV cells might result in a global shift in thiol oxidation and a concomitant decrease in phosphatase activity. To monitor global thiol oxidation we used an *in situ* reverse labeling assay. Reduced thiols were first alkylated with IAA, whereas oxidized residues are protected from this alkylation. Cells are then treated with a strong reductant to reduce all oxidized thiols. The newly reduced nonalkylated thiols are subsequently labeled with *fluorescently tagged 5-IAF*. 253J-BV cells display an increase in 5-IAF labeling relative to the 253J cells, indicating a general increase in thiol oxidation formation (Fig. 5A) which was further enhanced by H2O2 treatment. Increased thiol oxidation was also accompanied by a global decrease in phosphatase activity of the 253J-BV cells, which was exacerbated by sublethal H2O2 treatment (Fig. 5B). These findings suggest that oxidative inactivation of phosphatases may contribute to the enhanced migratory signaling activity of metastatic 253J-BV cells.

The primary phosphatase responsible for regulation of p130Cas phosphorylation is PTPN12 (23). The importance of PTPN12 in regulating p130Cas phosphorylation and migration was shown when expression of PTPN12 was decreased in the nonmetastatic 253J cells. An approximately 25% knockdown of PTPN12 dramatically increased the level of p130Cas phosphorylation in 253J cells (Fig. 5D). This moderate knockdown resulted in statistically significant increases in 253J cell migration (Fig. 5D). These data...
Figure 3. Intracellular redox status regulates p130Cas cellular localization. A, p130Cas is more abundant in the membrane/cytoskeletal fraction of 253J-BV cells than 253J cells. H2O2 treatment (10 minutes) enhances p130Cas membrane recruitment as assessed by membrane/cytosol fractionation. B, quantification of p130Cas immunoreactive protein in membrane fractions normalized to GAPDH levels. C, immunofluorescent staining reveals enhanced membrane distribution of phospho-p130Cas in metastatic 253-BV compared with 253J cells. Cells were fixed and stained for phospho-p130Cas Y165 (Alexa-Fluor 488, green), nucleus (DAPI, blue), and F-actin (Phalloidin Texas Red). Following mounting, images were acquired using Zeiss AxioObserver.Z1 with AxioCamM3, Apotome (63x/1.25NA oil objective; scale bar, 10 μm). D to G, phospho-p130Cas localization at the leading edge is dependent on redox status of cells. 253J or 253J-BV cells were plated on coverslips, allowed to reach a confluent monolayer and monitored for migration following scratch wounding in serum-free media, with or without 50 μmol/L H2O2 (253J, D and E) or 500 U/mL CAT (253J-BV, F and G). After 6 hours, cells were fixed and immunofluorescently labeled for phospho-p130Cas Y165 (Alexa-Fluor 488, green), nucleus (DAPI, blue), and F-actin (Phalloidin Texas Red). White arrows point to examples of phospho-p130Cas-positive protrusions. Images were taken of cells at the wound edge migrating into the direction of the opposing leading edge of cells (indicated by red arrows), using Zeiss AxioObserver.Z1 with AxioCamM3, Apotome (63x/1.25NA oil objective; scale bar, 10 μm). H, quantification of the number of phospho-p130Cas-positive protrusions (6–10 images per treatment quantified, with a range of 5–20 protrusions per image; one-way ANOVA; * * * * P < 0.01; * * * * * * * * P < 0.001).
indicate that decreases in PTPN12 activity either by oxidation or direct suppression can dramatically influence migratory signaling by maintaining p130Cas.

Discussion

In the present study, we show that subtle increases in steady-state H2O2 levels within metastatic bladder cancer cells are sufficient and necessary to drive promigratory signaling via regulation of p130Cas. These changes are all reliant on subtle picomolar increases in endogenous H2O2 levels within the metastatic variant cells (i.e., 253J: 18 pmol/L; 253J-BV: 32 pmol/L) without prior treatment or stimulation of cells (10). We also show that intracellular shifts in steady state H2O2 production result in detectable changes in promigratory signaling and further shed light on a redox-signaling network that may play an important role in metastatic disease progression.

The role of intracellular H2O2 was validated in all cell lines when coexpression or treatment with recombinant CAT effectively abrogated these prometastatic phenotypes and signaling cascades (12, 24). These data also suggest that antioxidant enzyme expression can have profound effects on promigratory cellular signaling pathways. Of note are markedly lower basal CAT expression levels previously reported in 253J-BV cells when compared with their parental control (10), which likely explains some of the robust H2O2-dependent signaling observed in these cells. It is interesting to note that changes in antioxidant expression profile correlate with metastatic disease. Increased Sod2 levels are commonly associated with more invasive disease and poor

Figure 4. p130Cas downstream signaling is redox dependent. A, intracellular redox status regulates Crk-p130Cas interaction. Cells were lysed using RIPA buffer, followed by immunoprecipitation (IP) with antibody against Crk and immunoblotting (IB) with indicated antibodies. CAT treatment (as in Fig. 2) abrogates association of Crk with p130Cas. B, Rac1 activation in 253J-BV cells is decreased following CAT adenoviral delivery or exogenous CAT treatment. GTP-bound active Rac1 was isolated from cell lysates using PAK-1–binding domain–conjugated agarose, as described in Materials and Methods. Total Rac1, phospho-p130Cas, and total p130Cas levels were assessed by IB of input. C, p130Cas knockdown significantly abrogates 253J-BV migration in a wound healing assay. Cells were mock transfected (–) with siRNA construct against p130Cas or scramble control before wound healing assay (n = 7; mean ± SEM; t test; *, P < 0.05; ***, P < 0.0001, compared with scramble control). Immunoblot for p130Cas shows efficiency of p130Cas protein knockdown. D, siRNA p130Cas knockdown decreases invasive properties of 253J-BV cells through Matrigel. Cells invaded through to the bottom surface of the transwell chamber were quantified (n = 3; mean ± SEM; t test; **, P < 0.01, compared with scramble control).
Figure 5. Enhanced intracellular redox status leads to increase in PTPN12 oxidation. A, total protein disulfide status of cells with or without 50 μmol/L H2O2 (10 minutes) was assessed using reverse in situ 5-IAF labeling as outlined in Materials and Methods. All reduced thiols residues were allylated using nonlabeled IAA. The remaining disulfides were reduced with DTT and acetylated using 5-IAF. Significant increases in in situ 5-IAF labeling were observed in 253J-BV cells compared with 253J cells using an Olympus Fluoview1000 Confocal Microscope. Average intensity units per cell were measured following background correction (n = 30; mean ± SEM; t test; *, P < 0.05; **, P < 0.01). B, total phosphatase activity is decreased in 253J-BV cells. Cells were treated with or without 50 μmol/L H2O2 for 10 minutes in serum-free media and the ability of cell lysates to dephosphorylate p-nitrophenol-phosphate was assessed colorimetrically. C, knockdown of PTPN12 in 253J cells increases p130Cas Y165 phosphorylation. Cells were mock transfected (-), with siRNA construct against PTPN12 or scramble control, and cell lysates subjected to immunoblotting with the indicated antibodies. D, PTPN12 knockdown significantly enhances migration of 253J cells in wound healing assays. Cells were transfected as in D, monitored for migration for 17 hours and percentage distance migrated by leading edge quantified (n = 4; mean ± SEM; t test; *, P < 0.05; **, P < 0.01, compared with scramble control).

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involved in p130Cas dephosphorylation is important in regulating cellular migration and focal adhesion assembly, specifically by regulating the activation of Rac-1, and has previously been shown to be under regulation by oxidation (23, 44–47). Furthermore, subtle decreases in PTPN12 levels had a profound impact tumor cell migration and p130Cas signaling (Fig. 5C).

The ability of cancer cells to migrate from their primary to a secondary site involves coordinating events that induce detachment, alter integrin engagement and allow migration through ECM. ROS through H₂O₂ regulate a multitude of cellular signaling cascades primarily due to their ability to reversibly oxidize thiolate anions of cysteine residues (1, 8). Many of the redox-sensitive signals control distinct aspects of the migratory and invasive phenotype. Lee and colleagues first identified the dual lipid phosphatase PTEN as reversibly sensitive to H₂O₂-dependent inactivation (48). PTEN plays a critical role in modulating phosphoinositide distribution during directed migration (49) and its oxidation by mitochondrial-derived H₂O₂ drives both the angiogenic and migratory phenotype (24). H₂O₂ generation has been shown to be targeted at focal complexes in lamellipodia and membrane ruffles through tethering NADPH oxidase family members via scaffolding proteins. This compartmentalized production of ROS is directed to tyrosine phosphatases that are enriched in these compartments (50, 51). Our prior work indicates that lamellipodia are also enriched in mitochondria and provide an additional source for migratory ROS production. Indeed, recent work using a mitochondrial-targeted CAT construct developed in our laboratory (52) indicates that mitochondrial ROS generation contributes to VEGF-dependent endothelial cell migration (53). We have also established that many of the key migratory signaling molecules that are engaged during endothelial cell migration also drive the invasive-ness of metastatic bladder cancer cells. Thus, the increase in steady state H₂O₂ associated with acquisition of the metastatic phenotype likely drives phosphatase inactivation leading to activation of FAK, Rho-related small GTPase Rac1 and mitogen-activated protein kinase (MAPK) signaling. However, a key difference is that these redox-driven signals are constitutively active in metastatic bladder cancer. It is our hypothesis that oxidants converge to drive the invasiveness of bladder tumors, thus opening a therapeutic window to limit metastatic disease progression using targeted-antioxidant based therapeutics.

These studies indicate that the ability to endure chronic oxidative stress is a feature acquired by metastatic cancer cells. Current neoadjuvant therapies for metastatic bladder disease display similar therapeutic efficacy and despite initial high response rates these treatments are not curative. Our preliminary work has identified imbalances in the redox-milieu as contributing to the metastatic phenotype. It is likely that oxidizing species impinge on a number of molecular targets that as a whole serve to engage metastatic disease progression. Thus, antioxidants may prove beneficial in limiting disease relapse when appropriately administered.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: N. Hempel, B. Mian, J.A. Melendez
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Hempel, B. Mian, J.A. Melendez
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