Research Paper

Intracellular redox status controls membrane localization of pro- and anti-migratory signaling molecules

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ABSTRACT

Shifts in intracellular Reactive Oxygen Species (ROS) have been shown to contribute to carcinogenesis and to tumor progression. In addition to DNA and cell damage by surges in ROS, sub-lethal increases in ROS are implicated in regulating cellular signaling that enhances pro-metastatic behavior. We previously showed that subtle increases in endogenous H2O2 regulate migratory and invasive behavior of metastatic bladder cancer cells through phosphate inhibition and consequential phosphorylation of p130cas, an adapter of the FAK signaling pathway. We further showed that enhanced redox status contributed to enhanced localization of p130cas to the membrane of metastatic cells. Here we show that this signaling complex can similarly be induced in a redox-engineered cell culture model that enables regulation of intracellular steady state H2O2 level by enforced expression of superoxide dismutase 2 (Sod2) and catalase. Expression of Sod2 leads to enhanced p130cas phosphorylation in HT-1080 fibrosarcoma and UM-UC-6 bladder cancer cells. These changes are mediated by H2O2, as co-expression of Catalase abrogates p130cas phosphorylation and its interaction with the adapter protein Crk. Importantly, we establish that the redox environment influence the localization of the tumor suppressor and phosphatase PTEN, in both redox-engineered and metastatic bladder cancer cells that display endogenous increases in H2O2. Importantly, PTEN oxidation leads to its dissociation from the plasma membrane. This indicates that oxidation of PTEN not only influences its activity, but also regulates its cellular localization, effectively removing it from its primary site of lipid phosphatase activity. These data introduce hitherto unappreciated paradigms whereby ROS can reciprocally regulate the cellular localization of pro- and anti-migratory signaling molecules, p130cas and PTEN, respectively. These data further confirm that altering antioxidant status and the intracellular ROS environment can have profound effects on pro-metastatic signaling pathways.

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Introduction

Redox signaling is implicated in regulating a diverse range of cellular functions. In diseases, such as cancer, sub-lethal increases in intracellular ROS have been associated with aberrant signaling that exsperates pathophysiological phenotypes. It has been shown that metastatic tumor cells display elevated in intracellular ROS and alterations in their antioxidant status [1–3]. For example, many aggressive cancers display increased mitochondrial manganese superoxide dismutase (Sod2) levels and loss of catalase (CAT) expression that are associated with stage and grade of disease progression and poor patient outcome. This shift in antioxidant enzyme levels may be an intrinsic adaptation of metastatic cancer cells to cope with changes in intracellular ROS [3]. A number of studies indicate that metastatic tumor cells have the capability to utilize these increases in ROS to regulate transcription and cellular signaling that contribute to metastatic disease progression [4–7].

We have developed number of cell culture models, which either display endogenous increases in intracellular ROS as a result of acquisition of the metastatic phenotype or through manipulation of antioxidant enzyme expression. We have observed that increased intracellular ROS levels can drive migration, invasion and metastasis in vitro and in vivo, and have begun to elucidate the redox-sensitive molecular triggers that accompany the metastatic phenotype [7–9]. Using a bladder cancer cell culture model of metastatic disease progression we have shown that a highly metastatic cell line derivative (253J-BV) relies on redox-mediated signaling to drive its migratory and invasive phenotype compared to a related non-metastatic parental cell line [7,10]. Subtle increases in the ROS milieu have been shown to have profound effects on pro-metastatic signaling of the Focal Adhesion Kinase (FAK) pathway by
enhancing signaling through the adapter protein p130cas (Crk-associated substrate) which links FAK to Rac-1 activation and cytoskeletal rearrangement during the process of migration and focal adhesion formation [7]. Redox regulation of signaling occurs primarily through reversible oxidation of proteins, which is particularly prevalent in the case of phosphatases, where active site cysteine residues are susceptible to oxidation due to their relatively low pKa [11]. Increases in endogenous steady state H2O2 enhance oxidation of the phosphatase pool, p130cas function and migratory activity of the metastatic 253-BV bladder cancer cells through the oxidative inhibition the p130cas inhibitory phosphatase, PTP-N12 [7]. In addition, ROS play a pivotal role in oxidative inactivation of the dual protein and lipid phosphatase PTEN (phosphatase and tensin homolog), leading to redistribution of the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) pools at the plasmamembrane, enhancing Akt signaling and tumor cell migration [12–14].

In the present study we show that redox-regulation of p130cas signaling is not limited to metastatic bladder cancer cells, but can similarly be induced by altering steady state H2O2 levels following manipulation of Sod2 and CAT expression. We previously used this model to show that Sod2-mediated changes in steady state H2O2 lead to an enhanced metastatic and angiogenic phenotype through PTEN oxidation and increased VEGF expression. We have also established that the Sod2-dependent increases in H2O2 drive high level expression of the matrix metalloproteinase MMP-1 that often accompanies metastatic disease progression [8,9,13,15]. By modulating steady state H2O2 levels using well characterized redox engineered fibrosarcoma cell lines [16], we observed similar changes in redox dependent p130cas phosphorylation and its membrane recruitment. Further, using this and a bladder cancer progression model, we demonstrate that intracellular redox status influences both PTEN oxidation and its cellular distribution. Oxidation moved PTEN away from its primary site of action at the cell membrane to the cytosol. These data show that increases in steady state H2O2 levels can have essentially opposing actions on membrane recruitment of pro- and anti-migratory signaling players, ultimately supporting a redox-dependent pro-metastatic signaling complex at the leading edge of metastatic tumor cells.

Results

Sod2 expression leads to H2O2-mediated p130cas phosphorylation

We previously showed that increases in both endogenous and exogenous-applied H2O2 can stimulate p130cas phosphorylation in 253 bladder cancer cells [7]. Analysis of redox engineered HT-1080 cells [16] revealed that Sod2 overexpression similarly enhanced p130cas phosphorylation, which was reversed by CAT co-expression (Fig. 1A). To confirm this finding, stable expression of Sod2 in another bladder cancer cell line UM-UC-6, enhanced phosphorylation of p130cas and FAK (Fig. 1B). Treatment of UM-UC-6 cells with exogenous CAT was also able to reverse these effects, suggesting that increases in H2O2 in response to Sod2 expression play a significant role in regulating phosphorylation of p130cas. In addition, Sod2 expression markedly enhanced Crk association with p130cas, which is dependent on p130cas phosphorylation and necessary for p130cas down-stream signaling. This association was again abrogated by CAT co-expression (Fig. 1C). These data show that modulating antioxidant enzyme levels similar to that observed in metastatic cells, can activate redox-dependent signaling networks that engage migratory activity.

Loss of p130cas prevents cell migration

We previously showed that Sod2 expression enhances migration and invasion of HT-1080 tumor cells [9]. To assess if p130cas is a

![Fig. 1. Sod2-mediated shifts in redox status regulate p130cas phosphorylation.](image)

![Fig. 2. p130cas knock-down significantly abrogates migration of HT-1080 redox engineered cells (stable-transfected with empty vector CMV, Sod2 or Sod2/CAT) in a wound healing assay. Cells were mock transfected (-) or with siRNA construct against p130cas or scramble control and allowed to reach a confluent monolayer. Migration of cells in serum-free media into the scratch wound was monitored for 24 h. Percentage of the distance migrated by the leading edge was quantified (n=7; mean ± SEM, t-test, **p < 0.01, ***p < 0.001, compared to scramble control).](image)
necessary component in regulating migration, p130cas expression was inhibited by siRNA. Migration of control (CMV vector) and Sod2-expressing cells was significantly abrogated following a decrease in p130cas expression (Fig. 2). Interestingly, the cells co-expressing CAT displayed little migratory activity and were unaffected by loss of p130cas, indicating that efficient H₂O₂ removal supersedes p130cas silencing and its ability to restrict migration.

Intracellular redox status regulates cellular distribution of p130cas to the membrane

Phosphorylation of p130cas by FAK and Src kinase is associated with p130cas membrane recruitment to the leading edge of migrating cells [17,18]. The presence of p130cas in membrane fractions was enhanced in cells stably expressing Sod2 and decreased upon co-expression with CAT (Fig. 3A). Levels of p130cas in membrane fractions were further enhanced upon treatment of cells with exogenous H₂O₂. The differential pattern of p130cas membrane localization was confirmed using immunofluorescence analysis (Fig. 3B). In Sod2 stable expressing cells, p130cas staining was observed more strongly in clear focal contact patterns compared to control (CMV vector) cells, indicative of membrane protrusions of migrating cells. Staining for phospho-p130cas was reduced and p130cas redistributed to the cytoplasm, following CAT co-expression (Sod2/CAT). This suggests that changes in intracellular H₂O₂, as a consequence of differential antioxidant expression, plays a role in the redistribution of p130cas to membrane protrusions of cells.

Increases in intracellular redox status enhance oxidation and inhibit membrane localization of the phosphatase PTEN

PTEN is a known tumor suppressor, which exerts its anti-migratory effects via both its lipid and protein phosphatase activities [19–21].

Fig. 3. Membrane localization of p130cas in redox engineered cells. (A) Membrane/cytoskeletal fractionation of cell lysates reveals enhanced p130cas localization to membrane fractions of Sod2 expressing cells, which is further enhanced by treatment of cells for 10 min with 500 μM H₂O₂, and abrogated by CAT expression. (B) Immunofluorescence staining reveals enhanced membrane distribution of phospho p130cas in redox engineered Sod2 expressing HT-1080 cells. Cells were fixed and stained for phospho-p130cas Y165 (Alexa Fluor 488, green), Nucleus (Dapi, blue) and F-actin (Phalloidin-Texas Red; Scale bar = 10 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
PTEN has been shown to negatively regulate the FAK pathway and enhance dephosphorylation of FAK and p130cas [22], and its activity can be regulated by oxidation [12–14]. We have previously established that Sod2 expression enhances PTEN oxidation using non-reducing SDS-PAGE to distinguish between its oxidized and reduced forms [13]. In the present study, we were able to visualize this difference by further stimulating the cells with exogenous H2O2 (Fig. 4A, lower left panels). Interestingly, when analyzing PTEN distribution following H2O2 treatment between membrane and cytosolic compartments, the fraction of oxidized PTEN was decreased in the membrane compartment compared to the oxidized fraction of PTEN in the cytosolic compartment of Sod2 expressing cells (Fig. 4A and B, A left panels). This suggests that oxidation not only leads to inactivation of PTEN, but also determines its cellular localization. As expected, cells co-expressing CAT displayed little overall PTEN oxidation and the presence of CAT abrogated the effects of exogenous H2O2 treatment. When analyzing PTEN localization on reducing SDS-PAGE it was apparent that the total pool of membrane associated PTEN was decreased in cells following H2O2 treatment (Fig. 4A, upper right panel). To confirm these findings we assessed the same patterns in our metastatic bladder cancer model [10]. Metastatic 253J-BV cells, which display an endogenous increase in intracellular H2O2, showed enhanced oxidation of PTEN compared to their non-metastatic parental 253J cells (Fig. 4C, lower left panel, cytosolic fraction). This was further enhanced following H2O2 treatment. The ratio of oxidized to reduced PTEN was higher in the cytosol than in the membrane fraction (Fig. 4C and D). Total PTEN levels at the membrane without H2O2 treatment were significantly decreased in the 253J-BV cells compared to those found at the membrane of the non-metastatic parental cells (Fig. 4C, right panel). In both cell lines total PTEN was further sequestered away from the membrane following H2O2 treatment (Fig. 4C and E, C right panels). PTEN is dependent on membrane association for its lipid phosphatase activity and this redox-dependent membrane dissociation represents a novel regulatory mechanism of PTEN. Further, these data have identified a new redox-signaling paradigm through membrane recruitment of distinct cellular signaling complexes. As such we have shown that endogenous or Sod2-mediated increases in H2O2 are able to drive the membrane recruitment of pro-migratory signaling players, such as p130cas, yet have the opposing effect on anti-migratory regulators, like PTEN.

**Discussion**

The role of ROS as novel signaling players during the process of metastasis is becoming increasingly apparent. As novel redox-regulated pathways are being elucidated the importance of spatio-temporal aspects of their regulation will be realized. As such, we show that subtle ROS changes can have profound and opposing effects on the membrane localization of pro- and anti-migratory signaling players and that changes in antioxidant expression may contribute to altering the activity of these signaling networks. In the present study, we confirm our previous findings that p130cas, an adapter protein linking FAK and Src kinase to the activation of Rac-1, is an important mediator of redox-dependent pro-migratory signaling. We show that manipulating steady-state H2O2 levels by Sod2 and CAT expression can alter p130cas phosphorylation and membrane recruitment. Interestingly, as we had previously

![Fig. 4. Oxidation leads to redistribution of PTEN from the membrane to the cytosol.](image-url)
observed in 253J-BV cells, Sod2 expressing HT-1080 cells also displayed slightly enhanced total levels of p130cas (Figs. 2 and 3). This suggests that the redox mediated increase in p130cas phosphorylation enhances p130cas protein stability [7]. Dephosphorylation has previously been shown to increase p130cas degradation [23] and regulation of p130cas phosphorylation by phosphatases appears to be an important regulatory mechanism of its function [18]. Given that many cancers display enhanced Sod2 expression with increasing stage and grade, enhanced signaling through p130cas may be a hallmark of these cancers, thereby increasing their metastatic phenotype. We previously showed that the redox-mediated increases in p130cas signaling are likely mediated through oxidation and inactivation of phosphatases, rather than at the level of its kinases Src and FAK [7,18,24].

The role of PTEN as a potent tumor suppressor is highlighted by the fact that many cancer types are characterized by a loss of PTEN expression. This dual lipid/protein phosphatase has been functionally related to the regulation of a plethora of signaling cascades, including those regulating migration. PTEN’s lipid phosphatase activity regulates the levels of PIP3 at the membrane and therefore influences PI3K/Akt signaling. A loss of PTEN results in enhanced PIP3 accumulation at the leading edge and this is associated with actin cytoskeletal re-arrangement [20,21]. PTEN’s lipid phosphatase activity has also been implicated in regulating the directionality of movement [21]. At the protein level, phosphorylation and oxidation are known mechanisms that regulate PTEN activity [14,25]. We have previously reported that alterations in the steady state levels of H2O2, through over-expression of Sod2, can result in increased oxidation and inactivation of PTEN [13]. This was reversed by effective removal of H2O2 following CAT expression. Concomitant with Sod2-dependent PTEN oxidation we saw accumulation of PIP3 at the plasma membrane [13]. PTEN’s protein phosphatase activity has also been implicated in negatively regulating migration. A loss of PTEN promotes migration via activation of Rac-1 and CDC 42 [26]. It is also thought that PTEN’s protein phosphatase activity is responsible for regulating epithelial to mesenchymal transition [19]. PTEN over-expression can decrease FAK and p130cas phosphorylation, implicating it as an important phosphatase in regulating p130cas signaling, in addition to PTP-N12 [22,27].

The distribution of PTEN is known to be spatially regulated and believed to be important for the differential distribution of PIP3 and PI(2)P at the leading and lagging edges, respectively [28]. Interestingly, like its anti-migratory activity, it has recently been shown that PTEN membrane recruitment is regulated by de-phosphorylation of its C-terminus, allowing for an “open” configuration and PIP3 binding [25]. Our data provide new evidence that oxidation of PTEN, like phosphorylation, restricts its membrane recruitment (Fig. 4). Whether oxidation leads to a conformational change that inhibits binding of PTEN to the membrane via PI(3)P remains to be investigated. One could speculate that an alternative scenario may involve the inhibition of PTEN phosphatase activity via oxidation of its active site cysteines, and a subsequent loss of PTEN to auto-dephosphorylate its C terminus. This would result in enhanced phosphorylation and a “closed” C2 domain confirmation, leading to loss of membrane association. The concept that subtle picomolar increases in intracellular H2O2 can essentially have opposing effects on membrane localization of pro- and anti-migratory signaling players, such as p130cas and PTEN, respectively, is extremely novel and adds another layer to the ever expanding role of ROS as regulators of cellular signaling.

Methods

Cell culture, treatments and transfection

Redox engineered human HT-1080 fibrosarcoma cells were created by stable expression of Sod2, Sod2 and cytosol-targeted CAT (Sod2/CAT) or empty vector (CMV) and cultured in MEM with 10% FBS, 1 mg/ml G418 and Pen/Strep [16]. Human urothelial carcinoma cells UM-UC-6 were stably transfected with eGFP or Sod2-eGFP constructs constructs and cultured in DMEM media containing 10% FBS, 1% MEM Vitamins, 1% penicillin/streptomycin solution, 1% sodium pyruvate, 1% amino acids, 1% l-glutamine, Pen/Strep and 0.5 μg/ml puromycin (Ye H. and Melendez J.A. et al., manuscript in revision). The highly metastatic bladder cancer cell line 253J-BV was derived from a poorly metastatic parental human bladder adenocarcinoma cell line, 253J, following five successive bladder xenografts [29]. 253J and 253J-BV cells were cultured in DMEM, with 4.5 g/l l-glucose, sodium piruvate and 10% FBS in a 37 °C humidified, 5% CO2 incubator [10].

For H2O2 (Sigma) treatments, cells were exposed to H2O2 at indicated doses and times in serum free DMEM. For catalase (CAT) treatments, cells were pretreated in 10% serum DMEM with 500 U/ml recombinant bovine CAT (Sigma) or 2 mM N-acetyl cysteine (NAC; Sigma) for 24 h. This was followed by treating the cells with the same concentrations in serum free media for an additional 18 h.

Antibodies, immunoblotting and immunoprecipitation

Crk and p130cas antibodies were purchased from BD Transduction Labs, Phospho-p130cas-Y165 and PTEN antibodies from Cell signaling, CAT and PY20 phospho tyrosine antibodies from Abcam, and GAPDH antibody from Ambion. Cleared cell lysates for co-IP and phospho-protein analysis were obtained using RIPA buffer lysis (150 mM NaCl, 50 mM Tris (pH 7.5), 1% NP-40, 0.5% deoxycholate, EDTA-free protease inhibitor cocktail, 1 mM sodium orthovanadate), followed by centrifugation (14,000 rpm, 15 min, 4 °C). Protein concentration was obtained using the BCA protein assay (Pierce/Thermo Scientific) and equal protein loaded on 4–12% NuPAGE (Invitrogen). For immunoprecipitation 1 mg of protein was incubated with 1 μg anti-Crk antibody (mouse IgG control) overnight at 4 °C, followed by incubation with protein G sepharose (30 μl). Proteins were eluted in 2x reducing SDS-PAGE sample buffer and electrophoresed, followed by transfer to nitrocellulose membranes (Invitrogen i blot transfer system). Primary antibody incubation was carried out in 5% BSA TBS-0.1%M-27 and 5% CO2 incubator [10].

Wound healing assays

siRNA constructs were transfected into cells using RNAi Max Lipofectamine reagent (Invitrogen). Briefly, 200,000 cells were transfected with either 100 pmol p130cas siRNA (Santa Cruz Biotechnol-ogy) or equal molar concentration of non-targeting siRNA (control #1, Thermo Scientific Dharmacon). Cells were grown to monolayer confluence and wounds created using a P200 sterile pipette tip. Cells were allowed to move in 0% serum media at 37 °C and monitored for movement by taking bright field images at time 0 and indicated time intervals. Images were obtained using a Zeiss Axio Observer.Z1 with an EC Plan-Neofluar 5x/0.15 M27 objective and AxiocamMR3. Distances were measured using Axiovision (Zeiss) software and subsequent image processing (ie marking of original wound edge) undertaken using Adobe Photoshop. Migration of cells was quantified as % distance migrated by leading edge.

Membrane/cytoskeletal fractionation

The membrane/cytoskeletal fractions were obtained using a protocol adapted from [30], as described in [7]. Briefly, cells were
washed in PBS, followed by lysis in ice-cold CSK buffer (0.5% Triton X-100, 10 mM TrisHCl (pH 6.8), 50 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM sodium orthovanadate and protease inhibitors). Following centrifugation (13,600 g, 30 min), the Triton X insoluble pellet (membrane/cytoskeletal fraction) was washed with CSK buffer, and boiled to resuspend in SDS buffer (1% SDS, 10 mM TrisHCl (pH 7.5), 2 mM EDTA, 1 mM sodium orthovanadate and protease inhibitors). Cell debris was collected by centrifugation at 13,600 g for 30 min at 4 °C and the supernatant Triton X insoluble fraction (membrane/cytoskeletal fraction) saved. Equivalent amounts of Triton X insoluble fraction in reference to protein concentration of the soluble fraction were loaded on 10% SDS-PAGE, followed by immunoblotting. For PTEN experiments, N-ethylmaleimide (NEM, 40 mM) was added to buffers to inhibit oxidation of the protein following lysis. Samples were run in non-reducing sample buffer to separate reduced and oxidized forms of PTEN on SDS-PAGE.

Immuno-fluorescence staining

Cells were seeded on glass coverslips, fixed with 4% paraformaldehyde/PBS, permeabilized with 0.1% Triton X 100/PBS and blocked in 3% FBS/PBS. Cells were incubated with primary antibody Phospho p130Cas Y165 at a dilution of 1:100 in 3% FBS/PBS, followed by incubation with anti rabbit Alexa-Fluor 488 conjugated secondary antibody (Invitrogen, 1:1000 in 3% FBS/PBS). F-actin was stained with Phalloidin TexasRed (Invitrogen, 1:50 dilution) and nuclear DNA with Dapi. Cells were mounted using Prolong Gold Antifade reagent (Invitrogen) and visualized on a Zeiss Axio Observer Z1 Fluorescence Microscope, using a 63 × 1.25 NA oil immersion objective and AxioCam MR3 with Apotome attachment. Image analysis was performed using Zeiss Axiovision software.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). T-tests and one-way ANOVA with Tukey’s Multiple Comparison Post-tests were performed using GraphPad Prism v6, GraphPad Software, San Diego California USA.

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