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Cysteine (C)-X-C Receptor 4 Regulates NADPH Oxidase-2 During Oxidative Stress in Prostate Cancer Cells

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Abstract Reactive oxygen species (ROS) are implicated in many human diseases, including cancer. We have previously demonstrated that ROS increased the expression and activity of the chemokine receptor, CXCR4, which enhanced metastatic functions in prostate cancer cells. Studies have also revealed that CXCR4 and its ligand, SDF-1α, promoted ROS accumulation; however the source of ROS was not investigated. Recent evidence suggested that ROS accumulation in prostate cancer cell lines was contributed by the NADPH oxidase (NOX) family of enzymes. Herein, we sought to determine whether the CXCR4/SDF-1α signaling axis mediates ROS production through NOX in prostate cancer. We observed an increase in intracellular ROS generation in prostate cancer cells upon SDF-1α stimulation compared to untreated samples. Conversely, lower levels of ROS were detected in cells treated with AMD3100 (CXCR4 antagonist) or the ROS scavenger, N-acetyl-cysteine (NAC). Markedly reduced levels of ROS were observed in cells treated with apocynin (NOX inhibitor) compared to rotenone (mitochondrial complex I inhibitor)-treated cells. Specifically, we determined that NOX2 responded to, and was regulated by, the SDF-1α/CXCR4 signaling axis. Moreover, chemical inhibition of the ERK1/2 and PI3K pathways revealed that PI3K/AKT signaling participated in CXCR4-mediated NOX activity, and that these collective signaling events resulted in enhanced cell movement towards a chemoattractant. Finally, NOX2 may be a potential therapeutic target, as Oncomine microarray database analysis of normal prostate, benign prostatic hyperplasia (BPH) and prostatic intraepithelial neoplasia (PIN) tissue samples determined a correlation between NOX2 expression and prostate cancer. Taken together, these results suggest that CXCR4/SDF-1α-mediated ROS production through NOX2 enzymes may be an emerging concept by which chemokine signaling progresses tumorigenesis.

Keywords CXCR4 · NOX2 · Prostate cancer · Reactive oxygen species · SDF-1α

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Introduction

Reactive oxygen species (ROS) are chemically reactive molecules derived from oxygen, namely, superoxide anion radical ($O_2^{-\cdot}$), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($^{\cdot}OH$) [1] . Under normal conditions, antioxidant enzymes, such as superoxide dismutase, peroxidase and catalase, aid in maintaining cellular redox homeostasis by removing or neutralizing ROS molecules [2]. However, an increase in the production of oxidizing species and/or insufficient levels of antioxidants can disrupt redox homeostasis, resulting in oxidative stress [3]. Due to their high reactivity, ROS are generally perceived as toxicants that induce various deleterious effects such as oxidative modifications to nucleic acids, proteins and other biomolecules, which can lead to cellular dysfunction, death or malignant transformation [4]. As a consequence, an accumulation of ROS contributes to the onset or progression of many diseases, including cancer [1]. However, depending on their nature and concentration, ROS molecules have also been shown to play an essential role in mediating protective mechanisms such as apoptosis, phagocytosis and

detoxification reactions, thus highlighting the complexity of these molecules [4]. Moreover, reactive oxidants have emerged as intracellular signaling molecules that regulate several pathways crucial to cancer development and progression [3]. As such, our laboratory demonstrated that increased cellular H_2O_2 led to increased expression and activity of C-X-C chemokine receptor type 4 (CXCR4), which resulted in proliferation, invasion, and migration of prostate cancer cells across an endothelial monolayer [5].

CXCR4 is a transmembrane, G protein-coupled receptor (GPCR), specific for its ligand, stromal cell-derived factor-1 alpha (SDF-1α). SDF-1α is a homeostatic chemokine that traditionally regulates hematopoiesis and leukocyte trafficking [6]. Nonetheless, CXCR4 is expressed in multiple cell types and tissues [7]. Since the initial report by Müller et al., where CXCR4 expression and signaling in breast cancer was shown to induce chemotaxis and invasion, and correlated with a poor prognosis [8], the CXCR4/SDF-1α signaling axis has been well established as a key mediator in the metastasis of many cancer types. Furthermore, CXCR4/SDF-1α signaling is associated with other malignant cellular processes, such as tumor progression, angiogenesis, and survival, correlating with stimulation of PI3K/AKT, ERK1/2, JAK/STAT, and other signaling pathways that progress cancer [9]. For these reasons, CXCR4 has become a major target for cancer therapeutics to attenuate intracellular signaling that is associated with metastasis [6]. In the absence of SDF-1α, we demonstrated that CXCR4 became active under oxidative stress, where H_2O_2 alone led to enhanced CXCR4 functions [5]. Contrarily, Sutton et al. revealed in human hepatoma cells that SDF-1α promoted an accumulation of ROS [10], which led to oncogenesis and survival. Determining whether activation of CXCR4 by SDF-1α brings about oxidative stress, however, has not been explored.

ROS produced in cells and tissues are derived from two major sources: (i) the mitochondrial electron transport chain and/or (ii) NADPH oxidases (NOX) [11]. While mitochondria generate ROS indirectly as a by-product of cellular metabolism, NOX enzymes produce ROS directly; to date ROS production is the only known biological function of NOX enzymes [1]. Initially observed in leukocytes, the NOX family of enzymes is comprised of 7 known members: NOX isoforms 1–5, DUOX1 and DUOX2 [1]. All members of the NOX family consist of six transmembrane domains, a NADPH-binding domain and a FAD-binding domain. Despite their structural similarities, NOX isoforms display distinct biochemical characteristics and subcellular localizations [12]. The first identified and most extensively studied isoform, NOX2 [1], is a multiprotein complex consisting of a membrane-bound, catalytic $gp91^{phox}$-$p22^{phox}$ heterodimer (flavocytochrome b_{558}), and cytosolic regulatory proteins $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and Rac [1]. Activation of NOX enzymes require translocation of the cytosolic regulatory proteins to the plasma membrane-bound flavocytochrome b_{558}, which, once collectively assembled, generates superoxide by transferring an electron from NADPH to oxygen, resulting in diverse biological functions from apoptosis to mitogenic signaling [1].

NOX enzymes have been implicated in cancer where Kumar et al. observed that increased ROS generation in prostate cancer cells, as compared to normal cells, was largely contributed by NOX enzymes [7]. Specifically, NOX2, NOX4 and NOX5 displayed the highest levels of ROS secretion, and their function was critical for the malignant phenotype of prostate cancer cells [7]. Likewise, other studies have detected NOX1, NOX2, NOX4 and NOX5 at higher levels in various cultured cancer cell lines and human tumors compared to normal tissues at early and late stages of tumorigenesis [11, 13], suggesting that NOX expression and function was context and tissue dependent. One such study indicated that NOX2-deficient bone marrow stem cells in mice exhibited impaired chemotaxis and invasion in response to SDF-1α, which was associated with diminished SDF-1α-mediated phosphorylation of AKT [14]. An Oncomine microarray database search determined a correlation between NOX2 expression and prostate cancer progression. Therefore, we sought to investigate the role of the SDF-1α/CXCR4 signaling axis in regulating NOX enzymes to further enhance prostate cancer progression. Using the human prostate cancer cell line, C4-2, as our model, we examined whether SDF-1α induced ROS accumulation via NOX enzymes, and whether this axis influenced CXCR4 tumorigenic functions. We found that ROS molecules were secreted from NOX enzymes, instead of the mitochondria, upon SDF-1α stimulation. Specifically, the SDF-1α/CXCR4 signaling axis regulated the NOX isoform, NOX2, as its expression was inhibited by AMD3100, a CXCR4 antagonist. Additionally, chemical inhibition of phosphatidylinositol 3-kinase (PI3K) decreased ROS accumulation, suggesting its participation in the signaling cascade. Finally, we observed that CXCR4-mediated wound healing was inhibited when NOX2 expression was repressed in prostate cancer cells, further supporting that CXCR4 and NOX2 correlate activities during oxidative stress.

Materials and Methods

Cell Culture, Antibodies and Reagents Conditions

C4-2 human prostate cancer cells were obtained from Dr. Valerie Odero-Marah, Center for Cancer Research and Therapeutic Development at Clark Atlanta University. C4-2 is an androgen-independent cell line developed by Chung et al. [15]. Cells were generated by first co-injecting a murine host with the human prostate cancer cell line, LNCaP, and fibroblasts from an osteosarcoma followed by castration and excision of the resulting tumor. Cells from the excised tumor were then co-injected with osteosarcoma fibroblasts into a

castrated mouse, yielding the C4-2 line [15]. Cells were maintained in complete media (RPMI 1640 containing 10 % fetal bovine serum (FBS), 1 % non-essential amino acids, 1 % antibiotic-antimycotic and 1 % L-glutamine) at 37 °C in 5 % CO_2, or starvation media (phenol-free RPMI containing 1 % L-glutamine) for 48-hours. All cells were maintained between 60 % to 80 % confluency. PD98059, N-acetylcysteine (NAC), anti-α-Tubulin, and 2,7′-dichlorofluorescein-diacetate (DCFDA) were from Sigma-Aldrich; LY294002 was from Cayman Chemicals; cell culture supplies were from MediaTech; SDF-1α was from PeproTech. The following human antibodies were from Cell Signaling: anti-AKT; anti-phospho-AKT (pAKT). Anti-NOX2 was from Santa Cruz.

Measurement of ROS

Cells (5×10^3 cells/well) were plated in black 96-well dishes in complete RPMI 1640 media for 24 h. After attachment, cells were incubated in starvation media for 48 h, followed by 1 h pretreatment with NAC (10 μM), AMD3100 (1 μg/ml), apocynin (200 μM) or rotenone (0.2 μM), then 3 h treatment with SDF-1α (100 ng/μl). ROS secretion was monitored using DCFDA (10 mM) prepared in phenol-free RPMI 1640 with a final concentration of 10 μM. After diffusion into the cell, DCFDA is deacetylated into a non-fluorescent compound, which was then oxidized by ROS, creating fluorescent 2′,7′-dichlorofluorescein (DCF). Using the Bio-Tek Synergy HT micro plate reader, DCF was detected by fluorescence spectroscopy with a maximum excitation and emission spectra of 498 nm and 522 nm, respectively. Intracellular superoxide production was measured using the total ROS/Superoxide Detection Kit (Enzo Life Sciences AG, Lausen, Switzerland). Briefly, C4-2 cells were plated in black 96 well plates. Following transfection with NOX2 siRNA and treatment with rotenone (0.2 μM) and/or SDF-1α (100 ng/μl), cells were washed once and incubated for 1 h in 100 μl of phenol-free RPMI containing 2 μM of oxidative stress detection reagent (green) and 2 μM of superoxide detection reagent (orange). Fluorescence was quantified using a fluorescence microplate reader and standard fluorescein (Ex=488 nm, Em=520 nm) and rhodamine (Ex=550 nm, Em=610 nm) filter sets.

PCR Amplification

Total RNA was isolated with Total RNA Kit-I (Omega Bio-TeK) as described by the manufacturer. Total RNA was reverse transcribed with M-MLV Transcriptase (Promega) to generate cDNA for PCR amplification. Specific sense and antisense primers (Table 1) for NOX 1, 2, 3, 4, 5 and housekeeping gene L19, were synthesized by Integrated DNA Technologies.

Western Blot Analysis

Cells (5×10^5 cells/well) were serum-starved for 48 h and incubated with appropriate treatments. Cells were washed with 1X PBS and lysed in buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1%Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM PMSF (Cell Signaling). Protein concentrations were estimated using Bradford Protein Reagent (BioRad). Equal concentrations of total cell lysate were resolved by SDS-PAGE and transferred to a polyvinylidene fluoride transfer membrane (PVDF). Non-specific binding sites were blocked with 5 % nonfat dry milk/0.1 % Tween-20/1X TBS (TBST), followed by incubation with primary antibodies for the proteins of interest in 3 % BSA-TBST (pAKT, AKT) or 5 % nonfat dry milk-TBST (NOX2). Membranes were washed in 1X TBST, and incubated in horseradish peroxidase-conjugated secondary antibodies (1:2000–1:5000; Jackson ImmunoResearch) in 3 % BSA-TBST or 5 % nonfat dry milk-TBST for 1 h at room temperature. Immunoblots were visualized using Super Signal West Pico Chemiluminescence reagent (Thermo Scientific).

Short Interfering RNA Transfection

Transfection of control and NOX2 specific short interfering RNA (siRNA-Santa Cruz) was carried out in C4-2 cells using Dharmafect 2 transfection reagent (Thermo Scientific). Cells (5×10^5) were plated in a 6-well dish and transfected with concentrations of 60–120 nM NOX2 or 60nM control siRNA in 10 % FBS/RPMI medium at 37 °C/5 % CO_2 for 24 h. Transfected cells were then harvested for western blot analysis, as previously described.

Plasmid Construct

C4-2 (pcDNA3-GFP-PTEN) and GFP (pcDNA3-GFP) constructs were generated as described previously [16]. Briefly, GFP (green fluorescent protein) was cloned into the 5′ end of pcDNA3 plasmid (Invitrogen), using *HindIII* and *BamHI* restriction enzyme sites to generate pcDNA3-GFP. The coding sequence for human PTEN was cloned into *BamHI* and *EcoRI* restriction sites of pcDNA3-GFP in order to generate pcDNA3-GFP-PTEN.

DNA Transient Transfections

Transient transfections were performed with 2 μg of concentrated DNA and jetPRIME® Polypus transfection, per the manufacturers' instructions. Briefly, C4-2 cells were incubated with jetPRIME®-DNA complexes in 10 % FBS/RPMI for 4 h and the media was replaced with 10 % FBS in RPMI for an

Table 1 NOX Isoform Primer Sequences

Target	Forward primer sequence (5′→3′)	Reverse primer sequence (3′→5′)
NOX1	TTGCAGATGAACAAGCGTGGCTTC	TCATTGTCCCACATTGGTCTCCCA
NOX2	TCACTGGAGTTGTCATCACGCTGT	AAGGGCCCATCAACCGCTATCTTA
NOX3	TGGCGCATTTCTTCAACCTGGAAC	TGCATACAAGACCACAGGGCCTAA
NOX4	AGCAGAGCCTCAGCATCTGTTCTT	AGCTTGGAATCTGGGCTCTTCCAT
NOX5	CTTTGCAGAGCGATTCTTTGCCCT	AACTGGATGTTCTGGTCCAGTGGT
L19	GAAATCGCCAATGCCAACTC	TCTTAGACCTGCGAGCCTCA

additional 24 h, prior to serum-starvation (24 h). Cells were then harvested for respective experiments.

Immunoprecipitation

Cells (1×10^6 cells/well) were seeded in p-100 tissue culture dishes, serum-starved for 48 h, followed by incubation with the appropriate treatments. Cells were harvested in 1X NP-40 lysis buffer containing 1 mM PMSF and 1X cocktail inhibitor (Roche). Five hundred micrograms (500 μg) of total protein was incubated with a NOX2 specific antibody overnight at 4 °C, then immunoprecipitated with protein A/G agarose beads for 2 h at 4 °C. Samples were centrifuged for 2 min at maximum speed, the supernatant removed and the bead pellet was washed with 40 μL of 1X NP-40 lysis buffer, followed by centrifugation for 2 min at max speed (twice) and a final washing in 1X PBS. Beads were boiled in sample buffer, proteins were separated by SDS-PAGE and western blot analysis was performed for pAKT and NOX2.

Scratch Wound

Cells were seeded into a 6-well culture plates and allowed to attach and form a confluent monolayer. A 10 μl pipette tip was used to scratch and remove cells from a discrete area of the confluent monolayer to form a cell-free zone into which cells at the edges of the wound can migrate. Cells were pretreated with NOX2 siRNA or LY294002, followed by SDF1α stimulation. Images of cell movement were captured at 24 and 48 h periods for data analysis at 10X magnification.

Microarray Database Mining

Data mining of published microarray databases was used to determine the relative expression levels of NOX2 in clinically relevant cancer cases of breast, head and neck, renal, lymphoma and prostate (see Table 2 for additional clinical information). The Oncomine (http://www.oncomine.org) database was queried for NOX2 and expression values for normal, benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and prostate carcinoma (PC) were represented as a Box and Whiskers plot. The Mann–Whitney test was used for comparison of each clinical group using GraphPad Prism statistical software (version 5).

Statistics and Quantifications

Data are presented as the mean±SE of at least three independent experiments. The data were analyzed for two-way ANOVA or Student *t*-test. All statistical analyses were done, and all graphs were generated using GraphPad Prism 5.0 software (GraphPad).

Results

CXCR4 Induced ROS Generation Through NOX Enzymes

SDF-1α induced free radical production in human hepatoma cells [2]. To determine whether this occurs in prostate cancer, stimulated C4-2 cells were stimulated with SDF-1α prior to measuring ROS secretion in the culture medium. As demonstrated in Fig. 1a, stimulation with SDF-1α resulted in a significant increase in ROS production compared to the untreated control, while lower levels of ROS were detected in cells treated with the CXCR4 antagonist, AMD3100. Similarly, cells pretreated with the ROS scavenger, N-acetyl-cysteine (NAC), secreted low levels of ROS. Likewise, SDF-1α did not stimulate significant ROS secretion when cells were simultaneously pretreated with the AMD3100 and NAC (Fig. 1a).

Next, we sought to identify the source of SDF-1α-mediated ROS secretion in prostate cancer cells. We evaluated the effects of various inhibitory agents (mitochondrial complex I inhibitor [rotenone] or NOX inhibitor [apocynin]) on ROS secretion in prostate cancer cells. As observed previously, SDF-1α stimulated ROS secretion in prostate cancer cells (Fig. 1b). ROS secretion was observed near basal (untreated) levels in cells pretreated with apocynin alone and together with SDF-1α (Fig. 1b). Cells pretreated with rotenone alone exhibited basal level ROS secretion as well; however, co-treatment with rotenone and SDF1-α increased ROS secretion, suggesting that the mitochondria was not the primary source of SDF-1α-mediated ROS production, but rather NOX enzymes.

Table 2 Microarray Clinical Data

Tissue Type	GEO accession	Age	Grade	Isolation method	Reference
Breast	GSE9014	44–71	N/A	Laser-Capture Microdissection	[40]
Invasive Breast Carcinoma	GSE9014	32–93	1–3	Laser-Capture Microdissection	[40]
Buccal Mucosa	N/A	N/A	N/A	Biopsy	[41]
Head and Neck Squamous Cell Carcinoma	N/A	42–84	1–3	Surgical Specimen	[41]
Renal Cortex	GSE14994	N/A	N/A	Tissue Specimen	[42]
Hereditary Clear Cell Renal Cell Carcinoma	GSE14994	N/A	N/A	Tissue Specimen	[42]
B-Lymphocyte	GSE60	N/A	N/A	B-Cells	[43]
Diffuse Large B-Cell Lymphoma	GSE61	N/A	N/A	Tissue Specimen	[43]
Prostate Gland	GSE6099	N/A	N/A	Laser-Capture Microdissection	[44]
Benign Prostatic Hyperplasia	GSE6099	N/A	N/A	Laser-Capture Microdissection	[44]
Orostatic Intraepithelial Neoplasia	GSE6099	N/A	N/A	Laser-Capture Microdissection	[44]
Prostate Carcinoma	GSE6099	N/A	3–5	Laser-Capture Microdissection	[44]

CXCR4 Regulated NOX2 Expression

To date, seven isoforms of NOX have been identified. To determine which NOX isoform was regulated by CXCR4, specific primers were designed for isoforms 1–5, and the mRNA was isolated and amplified by RT-PCR. We observed that CXCR4/SDF-1α signaling had no effect on NOX isoforms, 1, 3, 4, and 5, as they were all uniformly expressed in the presence and absence SDF-1α and/or AMD3100 (Fig. 2a). Interestingly, the NOX2 isoform appeared to be regulated by CXCR4/SDF-1α signaling, as indicated by an increase in NOX2 mRNA expression in the presence of SDF-1α and a decrease in expression upon treatment with AMD3100 (Fig. 2a). To further substantiate these results, we assessed NOX2 protein expression by immunoblot analysis. NOX2 protein expression patterns positively correlated with mRNA levels, where expression was enhanced by SDF-1α, and repressed by AMD3100 (Fig. 2b).

CXCR4 Induced Superoxide Generation Through NOX2

We determined that: (i) CXCR4-mediated ROS production was primarily through NOX enzymes and not the mitochondria; and (ii) of the seven NOX isoforms, NOX2 was regulated

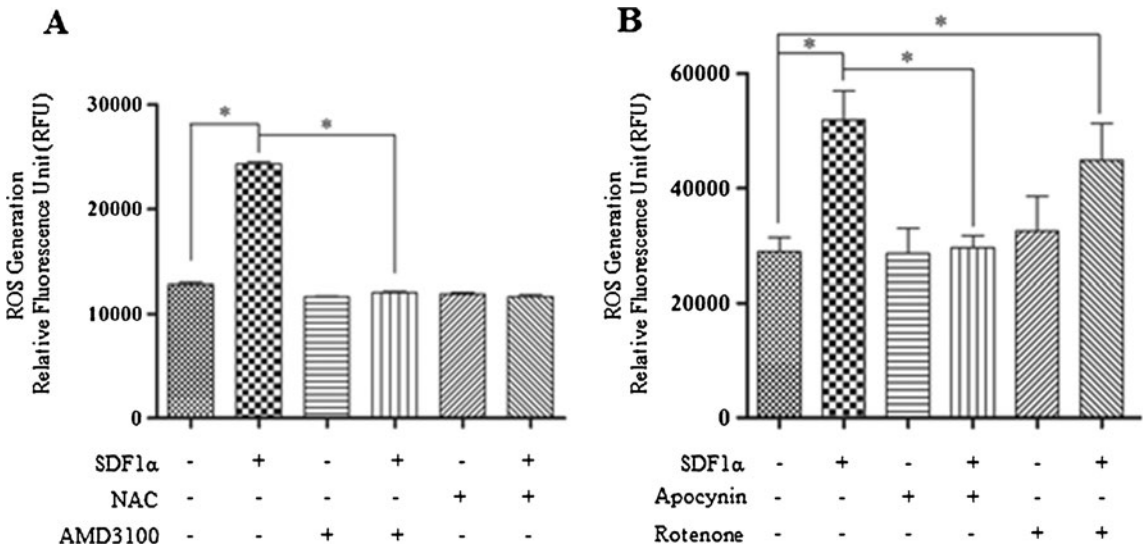

Fig. 1 CXCR4 induced ROS generation through NOX enzymes. **a** C4-2 cells were pre-treated with NAC (10 μM) or AMD3100 (1 μg/ml) for 1 h, followed by treatment with SDF-1α (100 ng/μl) for 3 h. Initial fluorescence readings were obtained prior to adding DCFDA for 15 min, and again immediately after. **b** C4-2 cells were pre-treated with apocynin (200 μM) or rotenone (0.2 μM) for 1 h, followed by treatment with SDF-1α (100 ng/μl) for 3 h. Readings for all assays were normalized to background (initial readings) and averages calculated. Fluorescence was detected using a micro plate reader, excitation=498 nm and emission=522 nm. *Error bars* reflect deviations from the mean for replicate measures. Statistically significant differences ($p<.05$) are indicated by *Asterisk*. Graphs shown are representative of triplicate experiments

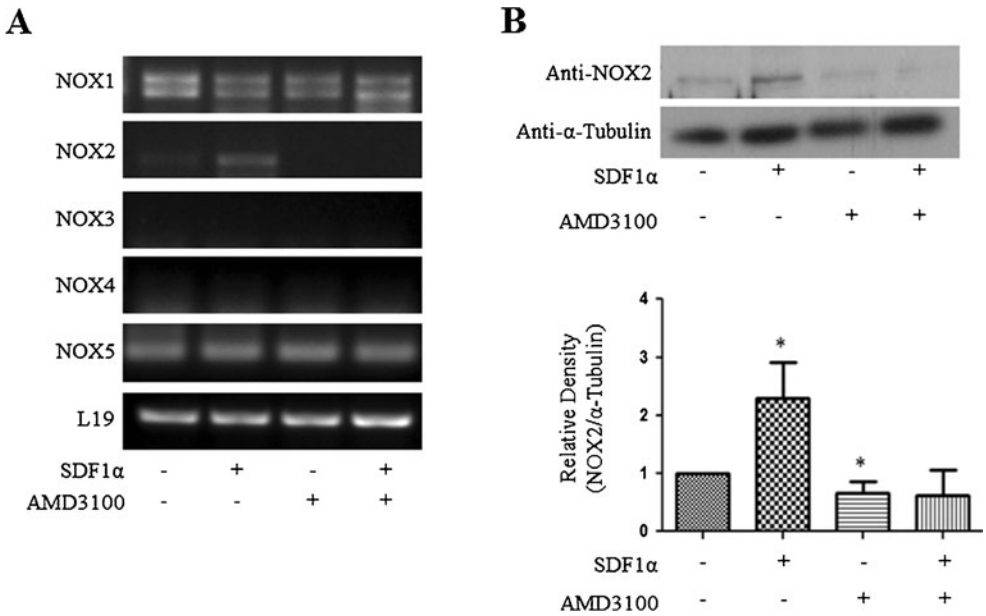

Fig. 2 CXCR4 regulated NOX2 expression. **a** An analysis of NOX isoforms 1–5 mRNA expression was performed. C4-2 cells were untreated or pretreated with AMD3100 (1 μg/mL) for 1 h prior to stimulation with SDF-1α (100 ng/μl) for 30 min. Two micrograms (2 μg) of total RNA was isolated with Total RNA Kit-I (Omega Bio-TeK) and reverse transcribed to cDNA for PCR amplification using specific primers (Table 1). L19 served as a loading control. **b** Western blot analysis and quantitative densitometry of NOX2 protein expression was performed. Cells were untreated or pre-treated with AMD3100, prior to stimulation with SDF-1α for 30 min. Forty micrograms (40 μg) of total protein was analyzed using a NOX2 specific antibody. α-Tubulin was the loading control. Autoradiographs shown are representative of triplicate experiments

by the CXCR4/SDF-1α signaling axis. Therefore, we sought to determine whether NOX2 was the *"specific"* isoform involved in CXCR4-mediated ROS production by diminishing its expression via short interfering RNA (siRNA). As seen in Fig. 3b, targeted silencing of NOX2 gene expression led to decreased intracellular generation of superoxide in C4-2 cells. NOX2-silenced cells stimulated with SDF-1α failed to elicit ROS production above basal level. Treatment with the mitochondrial complex I inhibitor, rotenone, had little to no effect on superoxide production. Co-treatment with rotenone and SDF-1α

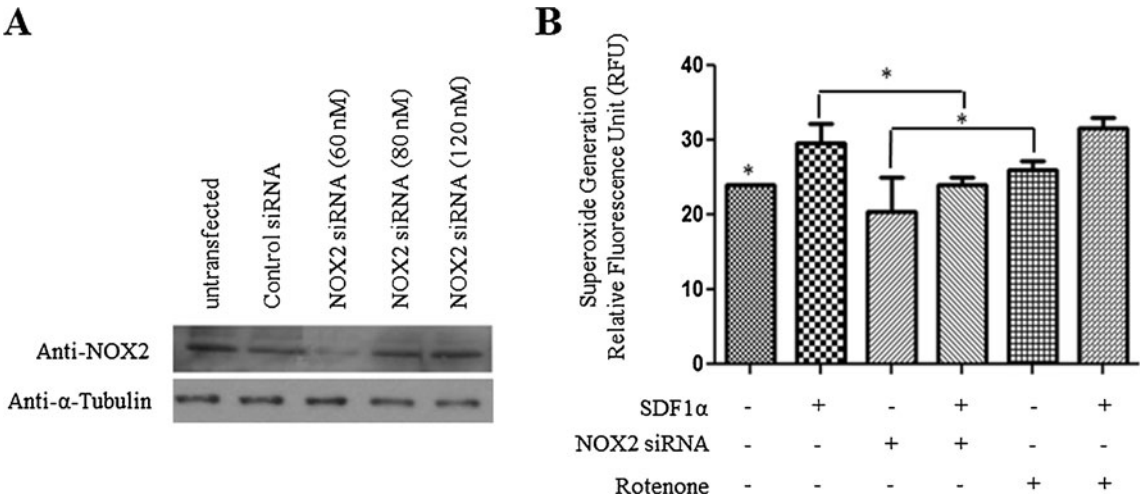

Fig. 3 CXCR4 induced superoxide generation through NOX2. **a** A dose–response (concentration) analysis of NOX2 siRNA was conducted at 60nM, 80nM and 120nM to determine the minimum concentration necessary for sufficient knockdown of targeted gene expression. Control siRNA (60nM) was utilized as a negative control to evaluate siRNA non-specific effects. **b** C4-2 cells were treated with NOX2 siRNA (60nM) or rotenone (0.2 μM) for 1 h, followed by treatment with SDF-1α (100 ng/μl) for 3 h. Readings for all assays were normalized to background (initial readings) and averages calculated. Fluorescence was detected using a micro plate reader, excitation=498 nm and emission=522 nm. Autoradiograph and graph shown are representative of triplicate experiments. *Error bars* reflect deviations from the mean for replicate measures. Statistically significant differences ($p<.05$) are indicated by *Asterisk*

increased ROS production. Taken together, these results suggest CXCR4-mediated intracellular ROS generation is primarily through NOX2.

CXCR4 Induced ROS Accumulation Through PI3K/AKT

Reactive oxygen species accumulation in cells has been linked to survival through activation of PI3K/AKT and ERK1/2 pathways [17]. Our lab previously demonstrated that expression of phosphorylated AKT (pAKT) increased in response to H_2O_2 in a dose dependent manner. Therefore, we investigated whether SDF-1α-mediated regulation of NOX2 enzymes also involved PI3K/AKT signaling. Our results revealed that treatment with the PI3K inhibitor, LY294002, decreased ROS secretion below basal levels in the presence of SDF-1α (Fig. 4). In contrast, cells treated with the ERK1/2 inhibitor, PD98059, and SDF-1α exhibited increased ROS secretion, suggesting that SDF1α-mediated ROS production was not dependent upon ERK1/2 signaling, but was instead, contributed, in part, by PI3K/AKT signaling (Fig. 4).

CXCR4-Induced Activation of AKT was NOX-Dependent

To further delineate the involvement of PI3K/AKT in NOX-mediated signaling, we treated cells with apocynin and assessed

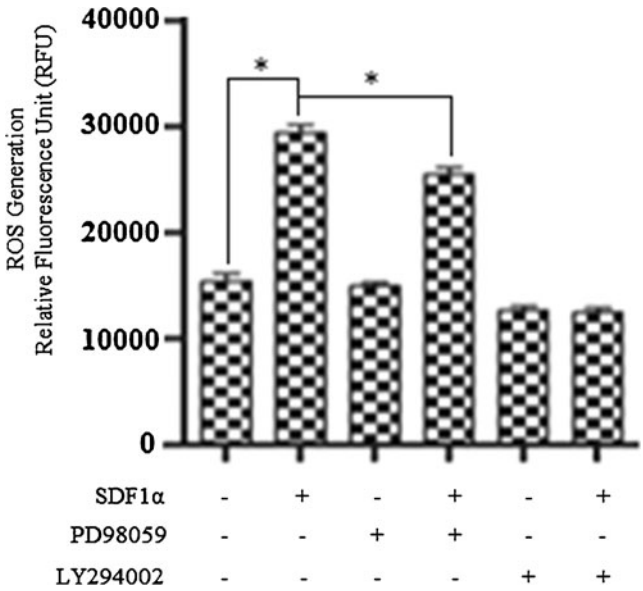

Fig. 4 CXCR4 induced ROS production through PI3K pathway. Cells were pre-treated with PD98059 (50 μM) or LY294002 (10 μM) for 1 h, followed by treatment SDF-1α (100 ng/μl) for 3 h. Initial fluorescence readings were obtained prior to adding DCFDA for 15 min, and again immediately after. Readings were normalized to background (initial readings) and averages calculated. Fluorescence was detected using a micro plate reader, excitation=498 nm and emission=522 nm. *Error bars* reflect deviations from the mean for replicate measures. Statistically significant differences ($p<.05$) are indicated by *Asterisk*. Graph shown is representative of triplicate experiments

its effect on the phosphorylation of AKT (pAKT). In cells where NOX activity was suppressed by apocycnin, we observed a concomitant decrease in pAKT compared to untreated cells (Fig. 5a). Likewise, apopcynin-pretreated cells expressed little pAKT when stimulated by SDF-1α (Fig. 5a). To determine whether NOX2 and pAKT physically associated in vitro, NOX2 expression was silenced via transient siRNA, followed by immunoprecipitation with a NOX2-specific antibody. Western blot analysis was then performed probing for pAKT protein expression. When compared to untransfected control, cells transfected with NOX2 siRNA alone exhibited decreased expression of pAKT (Fig. 5b). Stimulation of NOX2-siRNA cells with SDF-1α did not recover expression of pAKT, further validating coordinated efforts between NOX2 and the SDF-1α/CXCR4 signaling axis.

Absence of PTEN Permitted NOX2-Mediated Activation of AKT

ROS molecules oxidize PTEN within its catalytic domain by forming a disulfide bond between Cys124 and Cys71 in the active site, thus inactivating its phosphatase function [18], without affecting overall expression [5, 18]. We, too, observed an increase in this inactive form of PTEN during oxidative stress [16]. As a result, ROS-mediated oxidation of PTEN led to enhanced PI3K signaling and subsequent phosphorylation of AKT [19], suggesting that ROS-mediated upregulation of signaling cascades may not only be due to diminished levels of PTEN, but catalytic inactivation of an important tumor suppressor. Based collectively on our previous studies involving oxidative stress and signaling cascades downstream of PTEN, we hypothesized that the absence of PTEN expression in the PTEN-null cell line, C4-2, allows phosphorylation of AKT via NOX2-derived ROS. To investigate, we transiently reconstituted PTEN expression (pcDNA3-GFP-PTEN), or GFP (pcDNA3-GFP) as a control, into C4-2 cells (Fig. 6a) and evaluated the effects on pAKT expression. We observed that in the presence of PTEN, pAKT levels remained consistent, likely due to oxidation of PTEN by NOX2-derived ROS (Fig. 6b, lane 6). However, in cells co-transfected with functional PTEN, or GFP, and NOX2 siRNA, SDF-1α did not stimulate pAKT expression. One plausible explanation is that reduced NOX2-derived ROS generation restored the activity of PTEN, resulting in decreased phosphorylated AKT.

CXCR4-Mediated Cell Migration was Through NOX2 Activity

Due to the important role of the CXCR4/SDF-1α axis in cancer metastasis, we investigated whether SDF-1α induced prostate cancer cell migration (movement) via an in vitro scratch wound assay. Compared to untreated cells, SDF-1α enhanced cell migration after 24 h incubation (Fig. 7). Conversely, 24 h

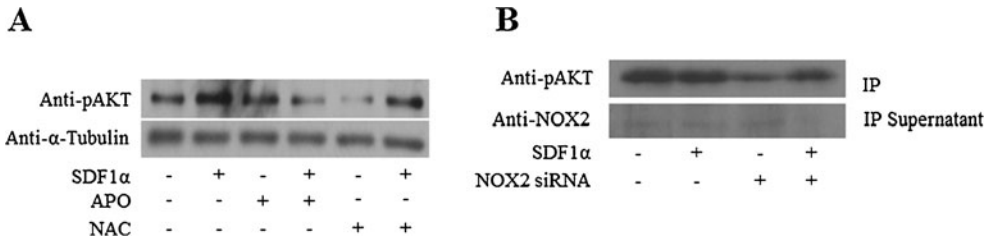

Fig. 5 CXCR4-induced activation of AKT was NOX dependent. **a** Cells were pretreated with apocynin (200 μm) and/or N-acetylcysteine (10 μm) for 1 h prior to SDF-1α (100 ng/μL, 30 min) stimulation. Forty micrograms (40 μg) of total protein were analyzed for pAKT expression by western blot analysis using a pAKT specific antibody. α-Tubulin served as a loading control. **b** Transiently transfected cells were treated with SDF-1α as above followed by harvesting for immunoprecipitation (IP) using a NOX2 specific antibody and protein A/G beads. Immunoblots were harvested for western blot analysis using a pAKT specific antibody. Autoradiographs shown are representative of triplicate experiments

treatment with the PI3K inhibitor, LY294002, resulted in negligible migration of prostate cancer cells. Likewise, diminished NOX2 expression in C4-2 cells prevented SDF-1α-mediated cell migration (Fig. 7). Collectively, our results demonstrate that the CXCR4/SDF-1α signaling axis along with NOX2 and the PI3K/AKT pathway coordinate efforts to progress prostate cancer during oxidative stress.

NOX2 Expression was Associated with Prostate Cancer Progression in Microarray Datasets

Analysis of NOX2 mRNA expression in cancer tissues was carried out using gene microarray datasets from the Oncomine database that compared cancer tissues to normal tissues. We found a significant ($P<0.05$) upregulation of NOX2 in microarray datasets from cancers of breast, head and neck, renal, lymphoma and prostate (Fig. 8a). Furthermore, NOX2 was significantly upregulated in prostate carcinoma compared to normal prostate cancer (Fig. 8b); however, no significant differences were observed comparing normal prostate to benign prostatic hyperplasia (BPH) and prostatic intraepithelial neoplasia (PIN) (Fig. 8b). These observations suggest that NOX2 may play a role in cancer development and progression and may be a reasonable therapeutic target.

Discussion

CXCR4 stimulation is known to enhance mitogenic functions, such as cell migration, proliferation, and invasion in cancer cells. Interestingly, these mitogenic functions are also promoted by ROS accumulation in a number of cancer cells, including prostate cancer. Oxidative stress has been implicated in cancer metastasis and progression by inducing expression of oncogenes, suppressing activity of anti-survival molecules, and by activating various cell survival and proliferation signaling pathways [20]. For instance, we demonstrated that H_2O_2 increased expression of CXCR4 in prostate cancer, leading to increased migration and invasion [5]. Furthermore, Sutton et al. reported that SDF-1α promoted an accumulation of ROS in human hepatoma cells, which also led to migration, invasion, and rapid cell growth [10]. Taken together, these studies suggest a

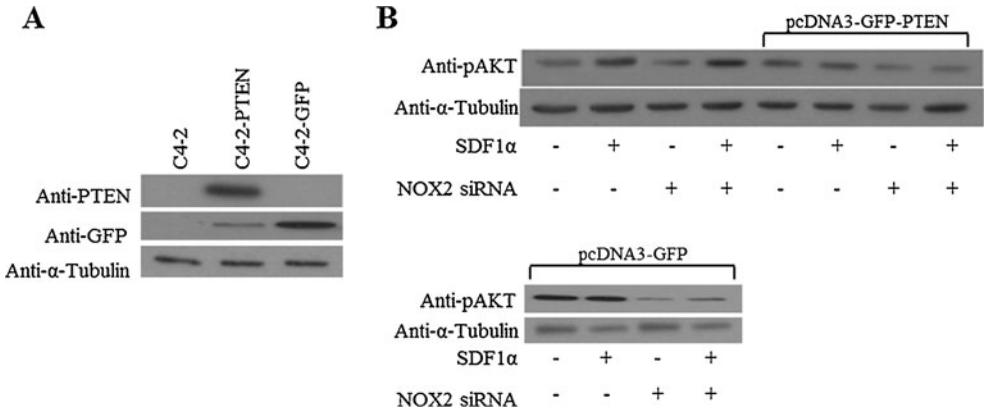

Fig. 6 Absence of PTEN permits NOX2-mediated activation of AKT. **a** Cells were transiently transfected with 2 μg of PTEN (pcDNA3-GFP-PTEN) or GFP (pcDNA3-GFP) plasmid DNA using jetPRIME® Polypus transfection reagent. To evaluate transfection efficiency, 40 μg of total protein was extracted from clones of transfected cells and analyzed by western blot analysis using anti-PTEN and anti-GFP specific antibodies. α-Tubulin served as a loading control. **b** Cells transiently transfected with PTEN, GFP and/or NOX2 siRNA were lysed and 30 μg of protein were analyzed for pAKT expression. α-Tubulin served as a loading control. Autoradiographs shown are representative of triplicate experiments

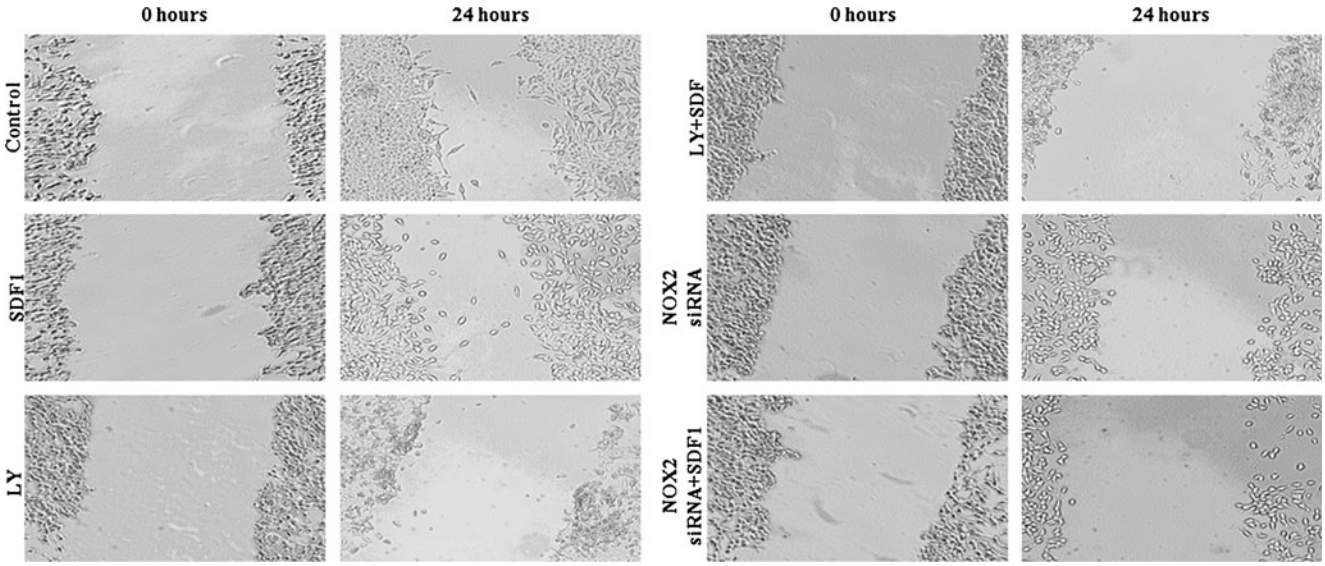

Fig. 7 CXCR4-mediated cell migration was through NOX2 activity. Cells were seeded into a 6-well culture plate and allowed to attach and form a confluent monolayer. Cells were transiently transfected with NOX2 siRNA. A scratch was made to remove cells from a discrete area of the confluent monolayer, forming a cell-free zone into which cells can migrate. Cells were pretreated with LY294002 (10 μM) for 1 h, followed by SDF-1α (1 ng/μl) stimulation. Images of cell movement were captured at 10x magnification at 0 and 24 h time points for data analysis. Images shown are representative of triplicate experiments

possible feedback mechanism between CXCR4 and ROS. Nonetheless, the endogenous sources of CXCR4/SDF-1α-mediated ROS production have yet to be addressed.

The signaling loop between SDF-1α chemokine, ROS molecules and CXCR4 expression and function is fairly new in cancer research. Models of ROS-mediated regulation of CXCR4 are scarcely described; however, Lee et al. demonstrated that depletion of ROS by N-acetylcysteine (NAC) led to CXCR4-mediated activation of AKT in B-cells [21]. Lin et al. described that CXCR4 induced ROS production in hematopoietic stem

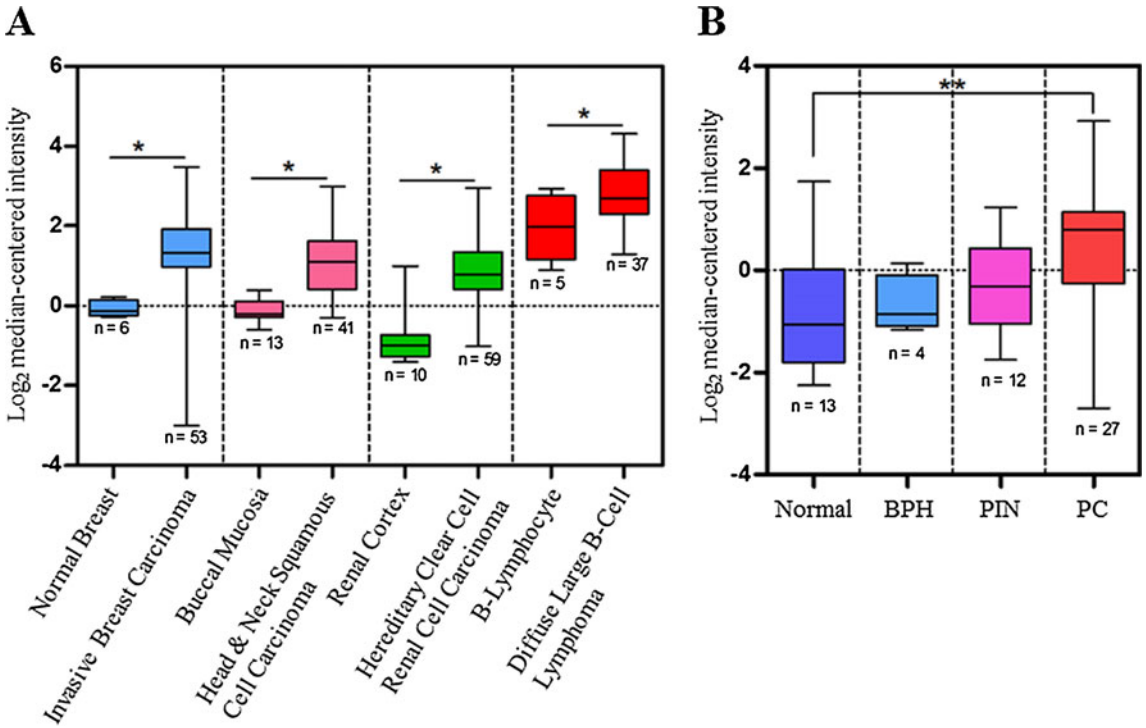

Fig. 8 NOX2 expression was associated with prostate cancer progression in microarray datasets. Box and whisker plots of NOX2 mRNA expression in (**a**) different cancer tissues and (**b**) prostate cancer stages using Oncomine. (* p-value<0.05)

cells [22]. Finally, Dar et al. described that loss of ROS reduced secretion of SDF-1α from human bone marrow osteoblasts and endothelial cells [23]. Likewise, we observed an increase in CXCR4 expression upon ROS treatment, which also correlated with increased expression of pAKT [24]. A number of studies have demonstrated that NOX-derived ROS may induce activation of AKT in a variety of cells, revealing AKT as a downstream target of NOX signaling [25]; there have been several proposed mechanisms to explain these events, such as concomitant protein-tyrosine phosphatase (PTP) inactivation and enhanced receptor tyrosine kinase (RTK) signaling [26]. Alternatively, it is also suggested that NOX-derived ROS may enhance AKT activation by catalytically inactivating the phosphatase, PTEN, which is a well-known negative regulator of the PI3K activity [27]. Furthermore, there is evidence that H_2O_2 accumulation in response to peptide growth factors in cells expressing NOX can transiently inactivate PTEN as a result of cysteine oxidation, leading to enhanced activation of the PI3K/AKT pathway [28]. Seo et al. provided evidence that in neuroblastoma cells, NOX inhibition diminished insulin-induced PI3K activation and AKT phosphorylation; this data supports our studies by suggesting that: (i) growth factors stimulated ROS accumulation from NOX enzymes; (ii) NOX-derived ROS participated in AKT activation; and (iii) a concomitant inhibition of PTEN by NOX-derived ROS was necessary to achieve a maximal response [29].

Several laboratories, including our group, have successfully demonstrated that ROS accumulation enhances prostate cancer cell migration and invasion in a CXCR4-dependent manner, which was associated with increased pAKT expression [24], and sometimes ERK1/2 [16]. A key concept that has been difficult to delineate is the order of regulation between ROS molecules and the CXCR4/SDF1α signaling axis. In accordance with these findings, we observed that while SDF1α-mediated ROS production was contributed, in part, by AKT, CXCR4-mediated phosphorylation of AKT was also NOX2-dependent.

Despite reports that the NOXs/DUOXs may be involved in maintaining optimal cellular redox levels, there is also accumulating evidence that NOX-derived ROS may elevate the risk for genomic instability and cancer by deregulating tumorigenic pathways, such as CXCR4 [30]. Cancer cells produce high levels of ROS, and in some cases, the source of these radical molecules has been linked to NOX/DUOX deregulation, as reported for prostate cancer (NOX1 and NOX5) [31, 32] and other tumor tissues. Two key studies by Lim et al. and Kumar et al., respectively, demonstrated that aggressive, malignant prostate tumors and cell lines were inherent to oxidative stress, and that the extra-mitochondrial NOX system was responsible for inherent ROS generation. NOX enzymes also conferred chemo- and radiation resistance in prostate tumors [33, 34].

Growth factor stimulation activates NOX enzymes at the membrane producing superoxide in the extracellular matrix, which is converted to a membrane-permeable molecule, H_2O_2, that mediates intracellular signaling events [35]. Studies have reported that tissues respond to mitogenic signals with an increase in ROS molecules, and that NOX enzyme expression increases in response to growth factors. For instance, growth factors PDGF and EGF stimulated H_2O_2 production in vascular smooth muscles cells and human epidermoid carcinoma cells [36, 37]. The increases in ROS molecules correlated with increased survival pathway activation and DNA synthesis, and were inhibited by antioxidants [36, 37]. In prostate cancer cells, exposure to androgen increased H_2O_2, as well as exposure to prostate specific antigen. Mitogenic signaling is also associated with increasing NOX mRNA, as we observed in Fig. 2a. In vascular smooth muscle cells, where NOX1 mRNA was lowly expressed, the growth factors PDGF and angiotensin II induced expression [38]. Lim et al. demonstrated that NOX1 protein and mRNA levels, and ROS molecule levels increased the tumorigencity of prostate cancer cells. What has yet to be examined is whether any of these mitogenic signals preferentially regulate a particular NOX enzyme to induce oxidative stress, and subsequently, the progression of tumors. Thus, understanding the relationship between known cancer promoting elements, such as CXCR4, ROS and NOX, will facilitate the development of more comprehensive therapeutics to enhance current clinical strategies to combat cancer metastasis [39].

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Conflict of Interest The authors declare that they have no conflict of interest.

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