NADPH oxidase NOX4 supports renal tumorigenesis by promoting the expression and nuclear accumulation of HIF2α

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Key words

Nox4, HIF-2α, superoxide scavenger, renal cell carcinoma
Abstract
Most sporadically occurring renal tumors include a functional loss of the tumor suppressor VHL.
Development of VHL-deficient renal cell carcinoma (RCC) relies upon activation of the hypoxia-inducible factor HIF-2α, a master transcriptional regulator of genes that drive diverse processes including angiogenesis, proliferation and anaerobic metabolism. In determining the critical functions for HIF-2α expression in RCC cells, the NADPH oxidase NOX4 has been identified, but the pathogenic contributions of NOX4 to RCC have not been evaluated directly. Here we report that NOX4 silencing in VHL-deficient RCC cells abrogates cell branching, invasion, colony formation and growth in a murine xenograft model RCC. These alterations were phenocopied by treatment of the superoxide scavenger, TEMPOL, or by overexpression of manganese superoxide dismutase or catalase. Notably, NOX4 silencing or superoxide scavenging was sufficient to block nuclear accumulation of HIF-2α in RCC cells. Our results offer direct evidence that NOX4 is critical for renal tumorigenesis and they show how NOX4 suppression and VHL re-expression in VHL-deficient RCC cells are genetically synonymous, supporting development of therapeutic regimens aimed at NOX4 blockade.

Introduction
Renal cell carcinoma (RCC) is a common adult malignancy with an estimated 65,150 new cases and 13,680 deaths in the United States in 2013 (1). Localized disease can be treated by surgical resection alone, but advanced RCC is notoriously resistant to cytotoxic therapy or radiation. Immunotherapy, the mainstay of treatment for several decades, is curative in fewer than 15% (2). Advances in the molecular genetics of kidney cancer have led to FDA-approval of targeted agents with good clinical response rates (3). However, complete, durable responses are rare, and novel therapeutic approaches are still desperately needed.

More than 80% of clear cell RCCs have lost or mutated both alleles of the von Hippel Lindau (VHL) tumor suppressor. VHL is the binding subunit of an E3 ubiquitin ligase complex that targets the alpha subunits of hypoxia-inducible transcription factors 1 and 2 (HIF-α) for ubiquitin-mediated, proteasomal degradation. In the absence of VHL, HIF-α accumulate in the cell, leading to increased transcription of more than 100
HIF-regulated genes involved in angiogenesis, anaerobic metabolism, proliferation, and other cell survival pathways. We and others have shown that HIF-2α is the relevant oncogenic target of VHL degradation. Forced accumulation of HIF-2α is sufficient to support xenograft growth of RCC cells despite reintroduction of wild type VHL, (4, 5) and specific HIF-2α inhibition suppresses tumor growth (6). In contrast, forced expression of HIF-1α suppresses xenograft growth, (4, 7), and specific HIF-1α shRNA enhances xenograft growth (8). HIF-1α and HIF-2α have unique, non-overlapping regulatory profiles suggesting a more pro-apoptotic rather than pro-proliferative role for the former (7, 9). In short, specific activation of HIF-2α appears to be critical for renal tumorigenesis.

We previously reported that HIF-2α expression and transactivation are dependent upon expression of the NADPH oxidase 4 (Nox4) (10). In the adult human, Nox4 is most highly expressed in the distal renal tubule where it generates intracellular superoxide and is implicated in oxygen sensing for regulation of erythropoietin, a HIF-dependent gene (11). In contrast to other Nox isoforms, Nox4 requires only p22phox for coactivation (12). In renal cancer cells, Nox4 is a major source of intracellular ROS (13). We hypothesized that this heightened oxidative state might promote HIF-2α transactivation under normal oxygen conditions. Consistent with this hypothesis, Nox4 silencing inhibits transactivation of VEGF, Glut-1, and erythropoietin by greater than 80% in 786-0 RCC cells. Furthermore, Nox4 siRNA suppresses HIF-2α and VHL at the mRNA and protein levels (10). Nox4-dependent expression of HIF-2α protein has been confirmed by others (14, 15).

Thus, HIF-2α is an established oncogene for clear cell kidney cancer and Nox4 is critical for its expression and transactivation in RCC. However, the contribution of Nox4 to renal tumorigenesis is not known. We report that in vitro branching morphogenesis and invasion are abrogated by Nox4 silencing and enhanced by Nox4 overexpression via generation of ROS and that in vivo RCC xenograft growth is suppressed by Nox4 silencing. Further, we report that Nox4 regulates the intracellular distribution of HIF-2α with abrogation of nuclear accumulation under both hypoxic and normal oxygen conditions.
**Materials and methods**

*Cell lines and cultures*  Established human conventional RCC lines, 786-0, RCC4 and Caki-1 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin and streptomycin. 786-0 (WT) and 786-0 (pRC), created by stable transfection of with wild-type VHL or empty pRC vector, respectively, were a gift from W. Kaelin (16). They were selected with G418 (500μg/mL) every sixth passage. RCC4 was generously provided by M. C. Simon and Caki1 cells were obtained from ATCC. Cell lines were routinely authenticated by DNA fingerprinting at the start and twice annually for the duration of these studies by the core University of Pittsburgh Cancer Institute Cell Culture and Cytogenetics Facility.

Stable Nox4 knockdown was achieved for each cell line by expressing two Nox4 shRNAs or a non-targeting shRNA in pSilencer™ 4.1-CMV puro (Ambion, Austin, TX) as previously described.(10) Stable transfectants were maintained in puromycin (1μg/mL). RT-PCR for Nox1-5, p22phox, p47phox and p67phox was performed as described (17). Adenoviral vectors Ad-EGFP, Ad-MnSOD and Ad-catalase were a generous gift of Dr. Yong Lee (18).

Adenoviral transduction was performed as previously described (19). Briefly, cells were infected at 100 or 200 MOI for 1.5 hours in DMEM. Assays were performed 48 hours post transduction. To overexpress Nox4, parental 786-0 cells were transfected with a pcDNA vector expressing the complete human Nox4 cDNA and antibiotic selection of stable clones was performed. Cells were pre-treated for 4 hours with indicated concentrations of DL-Dithiothreitol (DTT, Promega, Madison, WI) or 4-hydroxy-TEMPOL (Sigma-Aldrich, St. Louis, MO) prior to fixation or live cell assay. Drug was maintained in the media throughout live cell assays.

*Quantitative RT-PCR*  Total RNA was extracted from 786-O, RCC4, and LNCap cells with TRIzol reagent and RNeasy Mini Kit (Qiagen, Valencia,CA). First strand cDNA was synthesized using iScript cDNA synthesis kit (BIO-RAD, Hercules,CA ). Gene-specific TaqMan Gene Expression Assays primer sets and Master Mix were used for quantitative PCR of NOX4 (Hs00418356), NOX1 (Hs00246589), and GAPDH (Hs99999905). Samples were then subjected to real-time PCR analysis using the ABI StepOnePlus real-
Time PCR System (Applied Biosystems, Carlsbad, CA). Relative mRNA expression of each transcript was normalized against GAPDH.

**Western blot** Protein was extracted as previously described (4). Equal amounts of protein were subjected to separation in a 4.5–15% Tris-HCl gel, and the resolved proteins were transferred to polyvinylidene difluoride membrane. The blots were probed with anti-Nox4 rabbit monoclonal Ab (1:2,000, Abcam, Cambridge, MA) or β-Actin Ab (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), followed by HRP-conjugated secondary Ab. Bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

**Measurement of NAD(P)H oxidase activity** Superoxide production from membrane fraction was determined by lucigenin chemiluminescence as described (20). Briefly, 20 μg protein was added to 200 μl of 50 mM phosphate buffer with 1mM EGTA, 150mM sucrose, and 100 μM NADPH. Lucigenin was added, and chemiluminescence read every 30 seconds for 20 min (SpectraMax Plus 384, Sunnyvale, CA) and expressed as relative light units (RLU)/mg protein. Alternatively, ROS were measured using 2'7'-dichlorofluorescin diacetate (Sigma-Aldrich) as described (21). Fluorescence at 530 nm was measured using a Wallac Victor 1420 multilabel counter (Wallac Oy, Turku, Finland).

**Branching and invasion assays** Branching morphogenesis assays were performed as previously described (22). Briefly, 60μl cells at 3.5 x10^5 cells/ml media were mixed 1:1 with Matrigel in six flat bottomed 96-well plates. After 30 min at 37°C, 120μl DMEM with 10% FBS and 40ng recombinant hepatocyte growth factor HGF (Sigma-Aldrich, St. Louis, MO) was added and cells were incubated an additional 72 hours before determining the percent branched cells per well. Invasion assays were done in 8 micron matrigel 24-well invasion chambers (BD, Franklin Lakes, NJ). In the top chambers, 1.5 x 10^4 cells were plated in 200 μl media in five replicate wells. The bottom wells contained 750 μl media with 20 ng/ml HGF. After 24 hours at 37°C, matrigel and cells were removed from the top of the filter with a cotton swab and the filter bottom was fixed and stained with Diff-Quick (IMEB Inc, San Marcos, CA). Stained
cells were photographed at 100X magnification and counted. Significance was determined by Student T-test.

**Immunofluorescence and confocal microscopy**  Five x 10^4 cells were plated on cover-glass in 24 well plates for 12 hours. They were then incubated 4 hours with indicated media at 21% O_2 or 1% O_2. Cells were then fixed in 4% paraformaldehyde for 15 min, washed three times with PBS, then permeabilized with 0.1% Triton X-100 in PBS solution for 15 min. Cover slides were blocked with 2% BSA for 45 min and stained by primary antibodies for 60 min. After 5 washes with 0.5% BSA, secondary antibody was applied for 60 min, slides were counterstained with DAPI for 30 seconds and mounted with gelvatol. Images were taken with a FluoroView 1000 II confocal microscope.

**Soft agar colony formation**  Cells were grown in 0.3% bacto-agar (BD, Franklin Lakes, NJ) on a cushion of 0.6% agar, in triplicate. Fresh 0.3% agar was applied weekly. After 30 days, colonies were photographed and counted at 40X magnification.

**Mouse xenograft assay**  One million viable cells as determined by trypan blue exclusion were suspended in 100 μl Hank’s buffered saline and injected subcutaneously per flank of four 6-week female SCID beige mice (Charles River Laboratories, Wilimington, MA). Tumors were measured twice weekly with digital calipers (VWR, West Chester, PA) in the two largest dimensions by a technician blinded to the genotype. Mice were euthanized at 14 weeks and tumors harvested.

**Immunohistochemistry**  Paraffin slides were deparaffinized and hydrated to deionized water prior to heat-induced epitope retrieval by Diva antigen retrieval buffer (Biocare Medical). Endogenous peroxidase was quenched with 3% Hydrogen peroxide for 10 minutes followed by TBS buffer for 5 minutes. In an Dako Autostainer Plus Stainer, slides were blocked 10 minutes with CAS block (Invitrogen) and incubated 60 minutes with anti-HIF-2α mouse monoclonal Ab 1:500 (Novus Biologicals) or anti-Nox4 rabbit polyclonal Ab 1:100 (Abcam). Slides were then rinsed twice with TBS buffer and incubated with Dako Envision Dual Link HRP (Dako North America) for 30 minutes followed by Dako +Chromagen for 10 minutes. After
several rinses with deionized water, slides were counterstained with Harris Hematoxylin for 10 seconds, rinsed with tap water, dehydrated, cleared, and coverslipped. All incubations were performed at room temperature.

**Statistical analysis**

Data were expressed as the mean ± SE for at least three independent experiments from separate harvests. Statistical analysis was performed using the student t-test. P values < 0.05 versus control group were considered significant.

**Results**

Nox4 shRNA selectively suppressed Nox4 mRNA and protein expression and abrogated NADPH-dependent superoxide generation.

We first assessed endogenous expression of Nox 1-5 and co-activators in three human clear cell RCC cell lines, RCC4, 786-0, and Caki1. RCC4 and 786-0 lack functional pVHL due to a mutation in the VHL gene, while Caki1 cells express wild-type VHL. By semi-quantitative RT-PCR, all three lines abundantly expressed Nox4 and the co-factor p22phox (Fig. 1). Nox2 was detectable in RCC4 and Caki1 cells but Nox3 was seen only in Caki1 cells. In contrast to a prior report (14), we did not detect Nox1 in any of our cell lines (Supplemental Fig. 1).

Silencing of Nox4 mRNA and protein by specific shRNA (KD) relative to scramble control shRNA (NS) was confirmed by quantitative RT-PCR and Western blot, respectively. Although Nox4 expression was decreased by greater than 70%, Nox2 was not affected by silencing (Fig. 2a-c). A corresponding loss of NADPH-dependent superoxide generation from the cell membrane fraction was measured by lucigenin assay following Nox4 silencing (Fig. 2d), consistent with its role as the major source of superoxide in these cells. Similar superoxide suppression was demonstrated by lucigenin in RCC4 and Caki-1 following Nox4 knockdown. Membrane fraction from both 786-0 and RCC4 demonstrated the highest superoxide generation (Supplemental Fig 3). In summary, Nox4 was the dominant NADPH oxidase in three RCC cell lines.
lines. Nox4 shRNA silencing effectively and selectively silenced Nox4 expression, leading to abrogation of NADPH-dependent superoxide generation.

*Nox4 silencing inhibited branching morphogenesis and invasion by VHL-deficient cells.*

We have reported that Nox4 is critical for expression and transactivation of HIF-2α in VHL-deficient RCC cells, suggesting that Nox4 silencing might phenocopy VHL reintroduction in these cells. To explore the impact of Nox4 expression on renal tumorigenesis, we first assayed two established phenotypes of VHL-deficient renal cells: branching morphogenesis and invasion across a basement membrane. RCC cells demonstrate exuberant branching following exposure to hepatocyte growth factor (HGF) and will migrate through a matrigel-coated filter membrane toward an HGF/SF gradient. These behaviors are believed to reflect invasive and metastatic potential and are completely abrogated by re-introduction of a wild-type copy of the VHL tumor suppressor (22). To test our hypothesis that Nox4 expression is required to support branching and invasion, we assayed VHL-deficient 786-0 and RCC4 cells expressing KD or NS. Nox4 knockdown decreased the fraction of branching cells by 83% (p<0.001) and 93% (p<0.01) relative to NS cells in 786-0 and RCC4 cells, respectively (Fig. 3a-c), consistent with a critical requirement for Nox4 expression. Similarly, Nox4 silencing decreased the number of invasive cells by 70% (p<0.001) and 82% (p<0.05) relative to NS in 786-0 and RCC4 cells, respectively (Fig. 3d-f). Again, Nox4 silencing recapitulated the wild-type VHL phenotype.

To determine if overexpression of exogenous Nox4 could conversely enhance branching and invasion, we transfected parental 786-0 cells with a pcDNA vector expressing the complete human Nox4 cDNA. Following selection, cells demonstrated altered morphology with smaller, rounded cells, but similar growth kinetics (Supplemental Fig. 2a-b). Although these changes may reflect effects of oxidative signaling on multiple pathways, they may also be attributed to increased oxidative stress. Quantitative RT-PCR confirmed a marked increase in Nox4 mRNA expression (data not shown), which was confirmed at the protein level by Western blot (Fig. 3g). Invasive cells increased nearly 2-fold (p<0.05) following expression of pcDNA-Nox4 relative to empty vector (Fig. 3h-j). We did not observe increased branching in
the Nox4 overexpressed cells, likely due to the overall morphologic changes noted above. Taken together, these results indicate a regulatory role for Nox4 on the invasive phenotype of RCC.

**Superoxide scavenging with Tempol treatment or expression of adenoviral-SOD recapitulated the effect of Nox4 silencing on branching and invasion.**

To determine if the impact of Nox4 on RCC behavior is mediated by generation of superoxide, we used two parallel strategies. TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) is a heterocyclic chemical oxidant that reacts with intracellular superoxide anion to form hydrogen peroxide. Treatment with TEMPOL has been shown to increase detectable hydrogen peroxide(23) and thus, although it decreases intracellular superoxide, it does not necessarily decrease the overall oxidative status of the cell. TEMPOL exposure (0.125-10mM) did not impact 786-0 or RCC4 cell viability by Cell Titre Blue assay. When we treated parental 786-0 and RCC4 cells, we observed suppression of branching comparable to Nox4 silencing (p< 0.01) (Fig. 4a). Similarly, treatment with TEMPOL decreased invasion in a dose-dependent manner (p< 0.01) (Fig. 4b).

Although the major biologic functions of TEMPOL have been attributed to its ability to scavenge superoxide anion, it is not a specific SOD mimetic. It also has catalase-like activity and can inhibit hydroxide generation via the Fenton reaction making it a general purpose redox cycling agent. To reduce these off-target effects, we also examined branching and invasion after transducing RCC4 and 786-0 cells with adenoviral vectors expressing the ROS scavengers, manganese superoxide dismutase (Ad-SOD) or catalase (Ad-catalase). Nox4-generated superoxide cannot cross cell membranes, but is rapidly dismutated by SOD to hydrogen peroxide which freely diffuses through the cell (24). Hydrogen peroxide is in turn reduced by catalase to water and oxygen. Ad-SOD alone markedly suppressed branching by 77% (p<0.01) and 96% (p<0.01) relative to Ad-GFP in 786-0 and RCC4 cells, respectively. Comparable suppression was seen with co-transduction of Ad-catalase, or with Ad-catalase alone (Fig. 4c). Invasion assays (Fig. 4d) again revealed suppression of invasion by Ad-SOD relative to Ad-GFP in both 786-0 and RCC4 cells (p<0.01 for both). Co-transduction with Ad-catalase further decreased invasion in both cell lines (p<0.01).
By DCF assay, Ad-SOD expression alone had minimal impact on detectable ROS relative to Ad-GFP-transduced controls (Fig. 4e). This likely reflects the fact that DCF predominantly measures hydrogen peroxide, which is increased by the antioxidant activity of MnSOD. Thus, DCF will underestimate the impact of specific superoxide suppression. In contrast, catalase is specific for scavenging hydrogen peroxide and it is notable that transduction with Ad-catalase alone or in combination leads to ROS levels comparable to those observed with Nox4-silenced KD cells, again demonstrating that Nox4 is a major source of ROS in these cells (Fig. 4e). Suppression of the superoxide burst following exposure to TEMPO was confirmed using a lucigenin chemiluminescent assay for NADP(H)-dependent superoxide generation from isolated cell membranes (Figure 4f).

To summarize, scavenging of intracellular superoxide by either TEMPO or expression of exogenous superoxide dismutase mimics Nox4 silencing with respect to suppression of branching and invasion of RCC cells. Further suppression by catalase, suggests that hydrogen peroxide may be an equally important mediator of Nox4 signaling for branching and invasion.

**DTT induction of superoxide promoted cell branching.**

Dithiothreitol (DTT) is a strong antioxidant. However, under physiological conditions, the thiol-mediated antioxidant reaction results in intracellular generation of superoxide (25). We treated our cells with DTT to determine if further induction of superoxide could enhance branching and invasion relative to parental 786-0 and RCC cells. DTT treatment proved to be quite toxic resulting in high cell death (Supplemental Figure 4). The number of invasive cells was decreased in a dose dependent fashion, likely due to overall cell attrition. Despite this, we observed a nearly two fold increase in the percent branching cells in both 786-0 and RCC4 cells (p=0.03 and p=0.017, respectively) (Fig. 4a), consistent with our hypothesis that superoxide mediates branching morphogenesis.

**Nox4 silencing blocked nuclear accumulation of HIF-2α.**
We have reported that expression of HIF-2α at the mRNA and protein level is suppressed by Nox4 silencing (10). Others have confirmed that HIF-2α protein expression is dependent upon Nox4 expression (14, 15). However, we have observed suppression in HIF-2α transactivation even at high HIF-2α protein levels with Nox4 silencing. To test our hypothesis that Nox4 further contributes to activation of HIF-2α, we used confocal microscopy to examine HIF-2α cellular localization in our Nox4-silenced 786-0 and RCC4 cells. Activation is a multi-step process that requires binding to ARNT, nuclear translocation, binding to CBP/p300 and other cofactors, and binding to promoter DNA. Under hypoxic conditions, this requires FIH-mediated hydroxylation of asparagine residues. As HIF-2α has been shown to be less dependent upon FIH than HIF-1α for activation,(26) we hypothesize that Nox4 provides an alternate activating signal for HIF-2α.

Figure 5a shows representative confocal images of HIF-2α localization. We observed a Nox4-dependent distribution of endogenous HIF-2α protein. 786-0 and RCC4 NS cells showed diffuse staining throughout the cytoplasm and nucleus under normal oxygen conditions. As expected, culture at 1% oxygen resulted in nuclear concentration of HIF-2α. However, Nox4 KD cells showed a striking nuclear exclusion of HIF-2α with granular perinuclear enhancement. Notably, this nuclear exclusion was seen under both normal oxygen (Supplemental Fig 5a-d) and hypoxic conditions, suggesting that for HIF-2α, there may be a superoxide requirement even for hypoxic activation. Consistent with superoxide as a signaling intermediary, pre-treatment with TEMPO or transduction with Ad-SOD in NS cells mimicked the HIF-2α distribution observed with Nox4 silencing. Conversely, superoxide induction with DTT rescued nuclear accumulation despite Nox4 silencing. Western blot analysis of isolated nuclear fractions confirmed a dose-dependent decrease in nuclear HIF-2α expression following TEMPO treatment, whereas nuclear HIF-2α expression increased with DTT treatment (Fig. 5b-c). These findings support a role for Nox4 generated superoxide in nuclear accumulation of HIF-2α.

Nox4 silencing inhibits colony formation and xenograft tumor growth.
We next sought to determine if Nox4 silencing would be sufficient to prevent tumor growth. Neoplastic cells exhibit anchorage-independent growth and can proliferate in the absence of exogenous growth factors. This classic behavior, measured by the ability to form colonies in soft agar was decreased by 94% (p=0.008) in 786-0 KD cells relative to NS (Fig. 6a). To determine if Nox4 expression is similarly required to support in vivo tumor growth, we established xenografts of our 786-0 KD and control cells in SCID beige mice. 786-0 cells form tumors in immunocompromised mice that are abrogated by re-introduction of wild-type VHL (16). Attempts to establish subcutaneous xenografts with RCC4 were unsuccessful. Tumor growth curves are presented in Figure 6b. Mice injected with VHL-deficient 786-0 cells expressing control shRNA (NS) developed palpable tumors by week five, whereas Nox4 knockdown (KD) tumors were not detected before week nine. Further, KD tumors were significantly smaller at 14 weeks than NS (mean cross sectional area 35 mm² vs. 105 mm², p<0.01). Metastases to the brain, lungs or abdominal organs were not observed in any mice. Interestingly, despite clear inhibition of tumor growth, KD tumors demonstrated similar growth kinetics to NS once established. We postulate that the delayed tumors represent selection and enrichment of a subpopulation of cells that have escaped silencing - an effect that would lead to underestimation of tumor inhibition. Consistent with this, immunohistochemical evaluation of the 14-week tumor explants revealed that protein expression of Nox4 and HIF-2α was as high in the KD tumors as control (Supplemental Fig 6). Regardless, this intermediate phenotype is consistent with our hypothesis that Nox4 silencing suppresses RCC tumor growth, and suggests that therapies designed to target Nox4 or intracellular superoxide may have efficacy in RCC.

Discussion
Loss of the VHL tumor suppressor, resulting in abundant HIF-α protein and increased expression of HIF transcription targets occurs commonly in sporadic clear cell RCC. We previously reported that expression and transactivation of HIF-2α in VHL-deficient RCC cells is critically dependent upon expression of Nox4 (10). In the present study, we show that Nox4 silencing inhibits morphogenesis, invasive potential, colony formation, and xenograft tumor growth of VHL-deficient human RCC cells. Superoxide scavenging by treatment with TEMPOL or overexpression of superoxide dismutase or catalase mimicked the effects of Nox4 silencing, indicating a role for generation of superoxide and hydrogen peroxide in the tumorigenic
RCC phenotype. Furthermore, Nox4 silencing abrogated nuclear accumulation of HIF-2α under both normal and hypoxic oxygen conditions demonstrating that Nox4 is an alternative activating signal for HIF-2α translocation. To our knowledge, these data provide the first evidence that renal Nox4 expression is critical to support the renal tumorigenic phenotype, suggesting that it may function as a renal oncogene.

HIF-1α and HIF-2α are both subject to VHL-mediated oxygen dependent degradation and recognize the same DNA response element. However, they have differential effects on gene expression such that a shift in balance toward HIF-2α predominance promotes cell survival, whereas HIF-1α predominance favors apoptosis (27). In kidney cancer, HIF-α expression is biased toward HIF-2α and inhibition of HIF-2α suppresses VHL -/- tumor growth (6, 28). We speculate that heightened HIF-2α transcriptional activity is due in part to constitutive, isoform-specific induction by highly expressed renal Nox4. In renal cells, HIF-2α activity is held in check only by VHL-mediated protein degradation, leading to catastrophic proliferation and tumor formation following the loss of VHL. Consistent with this hypothesis, Nox4 silencing in our VHL-deficient RCC cells phenocopied re-expression of wild-type VHL.

Our results are consistent with other reports indicating that Nox4 modulates cellular phenotypic changes, including migration, invasion, and the epithelia-to-mesenchymal-transition (EMT). A recent report by Boudreau et al. found that inhibition of Nox4, either by shRNA knockdown or expression of a dominant-negative form, abrogated wound healing and cell migration in breast cancer epithelia (29). Similar to the results from this study, Yamaura et al. demonstrated that inhibition of Nox4 by siRNAs in melanoma cells decreased anchorage-independent growth and xenograft tumor growth in vivo (30). In keratinocytes, cell migration was inhibited by both diphenyliodonium (DPI), a non-specific flavoprotein inhibitor of Nox-enzymes, and Nox4 silencing (31). Nox4 has been implicated in the regulation of cell migration in non-tumorigenic cell types as well including myofibroblasts, endothelia, and vascular smooth muscle cells (32-34).

Although several sources of intracellular ROS exist, we and others have shown that Nox4 is a major producer of ROS in renal tumors (13). Importantly, there is growing evidence to suggest that ROS, in
conjunction with Nox expression, can induce the tumorigenic phenotype via heightened oxidative stress. For example, ROS, as well as Nox4, is required for invadopodia formation, an important step in invasion and metastasis that acts as a catalyst for extracellular matrix degradation (35, 36). Furthermore, ROS have been shown to regulate the production of matrix metalloproteinases (MMPs), critical proteolytic enzymes of tumorigenic invasion, in several tumor types including tumors of the pancreas and breast (37, 38) and glioblastomas (39).

ROS-induced, stabilization and induction of HIF-α has been described in a number of systems. Exogenous hydrogen peroxide stabilizes HIF-1α protein in normoxia (40). Expression of exogenous MnSOD in MCF-7 cells suppresses hypoxic accumulation of HIF-1α protein (41). MnSOD siRNA in these cells increases both detectable superoxide and HIF-1α accumulation, whereas TEMPOL decreased accumulation, implicating superoxide as the molecular effector (42). Quercetin, a potent flavonoid antioxidant induces normoxic accumulation of HIF-1α that is reversed by iron chelation, leading investigators to conclude that it inhibited HIF-α ubiquitination by inactivating the HIF prolyl hydroxylase (PHD) and preventing binding of pVHL (43). Redox-mediated inactivation of PHD was also described in pulmonary artery smooth muscle cells where overexpression or induction of Nox4 stabilized HIF-2α in the setting of decreased HIF-α hydroxylation and pVHL binding (44).

The mechanism of Nox4 regulation of HIF-2α in VHL-deficient RCC cells, however, remains unclear. Block et al describe increased expression p22(phox), leading to inactivation of tuberin and downstream ribosomal activation consistent with a translational pathway to increased HIF-2α accumulation (45). Our finding of altered nuclear expression of HIF-2α with Nox4 silencing or treatment with superoxide scavengers suggests an additional role for redox signaling on nuclear translocation or DNA binding. We speculate that redox signaling may alter the HIF-2α transcriptional complex and subsequent transactivation of target genes. Our study is limited by dependence upon established RCC cell lines and by potential off-target effects of shRNA. However, suppression of the cancer phenotype was reproducible in two VHL-deficient lines with independent shRNA sequences supporting our conclusion that experimental effects were due to specific Nox4 knockdown. Although we demonstrate that Nox4
silencing and Tempol treatment lead to specific superoxide suppression, our results cannot exclude the possibility that Nox4 signaling is also mediated by direct generation of hydrogen peroxide.

In summary, we show that Nox4 plays a crucial permissive role in the tumorigenic phenotype of VHL-deficient, human RCC cells and that specific Nox4 suppression inhibits intracellular superoxide generation, prevents nuclear accumulation of HIF-2α, and phenocopies re-expression of wild-type VHL.

Furthermore, Nox4 silencing is mimicked by superoxide scavengers, TEMPO or MnSOD or by catalase implicating superoxide anion and hydrogen peroxide as mediators of Nox4 regulation of HIF-2α. Taken together, these support an oncogenic role for Nox4 in conventional RCC and suggest that agents designed to target Nox4 might have clinical efficacy against kidney cancer.

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Figure Legends

Figure 1. Semiquantitative RT-PCR for NADPH oxidase family members and co-factors was performed in three human clear cell renal cancer cell lines, 786-0, RCC4 and Caki-1. The anticipated band sizes for Nox1-5 are 247, 413, 458, 286, and 630 bp, respectively, and co-factors p22phox, p47phox, and p67phox are 320, 771, and 760 bp, respectively. Beta-actin serves as an internal control.

Figure 2. (a) Semi-quantitative RT-PCR for Nox isoforms 2-4 in 786-0, RCC4 and Caki-1 cells transfected with Nox4-specific siRNA (KD) or non-targeting siRNA (NS). Beta-actin serves as an internal control. (b) Representative quantitative RT-PCR for Nox4 mRNA relative to beta-actin in RCC4 cells expressing Nox4 shRNA2 (KD) or scramble (NS). Columns, mean; bars, ± SE; *, P < 0.01 relative to NS. (c) Western blot analysis for Nox4 protein in three RCC cell lines. 786-0 indicates parental 786-0 cells (ATCC) in contrast to the 786-0 pRC (16) cells used for NS/KD. The upper band runs at the predicted 67kD for full length Nox4. (d) Lucigenin chemiluminescent assay for NADP(H)-dependent superoxide generation from isolated cell membranes of 786-0 NS and KD cells. Luminescence following addition of lucinigen is measured every 20 seconds for 20 minutes and expressed as relative light units (RLU).

Figure 3. Branching assays (a-c). VHL-deficient cells (786-0 or RCC4) expressing (a) control vector (NS) or (b) Nox4 shRNA (KD), cultured 72 hours in matrigel with HGF (0.33μg/ml). Bar graph (c) depicts the mean percent of branched cells. Invasion assays (d-f). VHL-deficient cells expressing (d) control vector (NS) or (e) Nox4-specific shRNA (KD) that have crossed a matrigel-coated, 8-micron pore filter toward an HGF gradient (magnification 100X). Small circles are pores. Bar graph (f) shows mean crossing cells. Exogenous Nox4 overexpression (g-j): Western blot for Nox4 expression in lysates of parental 786-0 stably transfected with pcDNA-Nox4 or empty vector. Beta-actin serves as an internal loading control. Invasion assays were performed as described above using pcDNA empty vector (h) or pcDNA-Nox4 (i) transfected cells. Bar graph (j) shows percent branching cells. Mean crossing cells are depicted in bar graph (k). Columns, mean; bars, ± SE; ** P < 0.01, * P<0.05.
Figure 4. Effects of reactive oxygen species scavengers and DTT on RCC cell branching and invasion. Branching assays (a-b) were performed as in Fig 3. Cells were treated with the indicated concentrations of TEMPOL or DTT (a) or transduced with adenoviral vectors expressing GFP (control), SOD, or catalase prior to matrigel culture (b). Invasion assays (c-d) demonstrate the number of cells crossing a transwell membrane in the presence of TEMPOL or DTT (c), or after transduction of Ad-GFP, Ad-SOD or Ad-catalase (d). Dichlorofluorescein assay (e) measures ROS produced by parental 786-0, 786-0 scramble (NS) or 786-0 expressing Nox4 shRNA (KD) cells transduced with adenoviral vectors expressing GFP (control), SOD, catalase, or both SOD and catalase. (f) Lucigenin chemiluminescent assay for superoxide on isolated cell membrane fraction of parental 786-0 cells with or without addition of TEMPOL. Columns, mean; bars, ± SE; * P < 0.02 relative to untreated or GFP controls.

Figure 5. Cellular distribution of HIF-2α. (a) Representative confocal microscopy images (magnification 400x) showing HIF-2α immunofluorescence, DAPI nuclear staining, or merged images. Cultures were grown in 1% oxygen for 8 hours prior to fixation and staining. Control (NS) or Nox4 shRNA (KD) cells were imaged following exposure to TEMPOL (0.25 mM), DTT (1mM) or transduction with Ad-SOD. Western blots (b and c) for HIF-2α protein expression in isolated nuclear fractions of 786-0 NS cells grown under normal oxygen conditions with serial dilutions of TEMPOL (b) or 786-0 KD cell grown under normoxic conditions with serial dilutions of DTT (c). TBP serves as an internal control for total loaded protein.

Figure 6. Nox4 expression is required for RCC cell colony formation and xenograft tumor growth. (a) Soft agar colony formation in 786-0 cells with Nox4 shRNA silencing (KD) or non-specific control shRNA (NS). Colonies larger than 20 μm were counted. (b) Mean cross-sectional area of tumor xenografts from four SCID beige mice per cohort injected subcutaneously with 786-0 NS or KD cells with or without re-expression of VHL. Tumor take rates were 100% for the 786-0 NS cohort and 75% for the KD cohort. Columns, mean; bars, ± SE; *, P < 0.008.
Figure 1

786-0

RCC4

Caki-1
Figure 3
Figure 5

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**a**

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NADPH oxidase NOX4 supports renal tumorigenesis by promoting the expression and nuclear accumulation of HIF2 α

Jennifer L Gregg, Robert M Turner II, Guimin Chang, et al.

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