NADPH Oxidase NOX4 Supports Renal Tumorigenesis by Promoting the Expression and Nuclear Accumulation of HIF2α

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Abstract

Most sporadically occurring renal tumors include a functional loss of the tumor suppressor von Hippel Lindau (VHL). Development of VHL-deficient renal cell carcinoma (RCC) relies upon activation of the hypoxia-inducible factor-2α (HIF2α), a master transcriptional regulator of genes that drive diverse processes, including angiogenesis, proliferation, and anaerobic metabolism. In determining the critical functions for HIF2α 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 HIF2α 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 α subunits of hypoxia-inducible transcription factors 1 and 2 (HIF1α and HIF2α) for ubiquitin-mediated, proteasomal degradation. In the absence of VHL, HIF1α 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 HIF2α is the relevant oncogenic target of VHL degradation. Forced accumulation of HIF2α is sufficient to support xenograft growth of RCC cells despite reintroduction of wild-type VHL (4, 5) and specific HIF2α inhibition suppresses tumor growth (6). In contrast, forced expression of HIF1α suppresses xenograft growth, (4, 7), and specific HIF1α shRNA enhances xenograft growth (8). HIF1α and HIF2α have unique, nonoverlapping regulatory profiles suggesting a more proapoptotic rather than proproliferative role for the former (7, 9). In short, specific activation of HIF2α seems to be critical for renal tumorigenesis.

We previously reported that HIF2α expression and transactivation are dependent upon expression of the NADPH oxidase 4 (Nox4; ref. 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 ofintracellular reactive oxygen species (ROS; ref. 13). We hypothesized that this heightened oxidative state might promote HIF2α transactivation under normal oxygen conditions.
Consistent with this hypothesis, Nox4 silencing inhibits transactivation of VEGF, Glt1, and erythropoietin by greater than 80% in 786-0 RCC cells. Furthermore, Nox4 siRNA suppresses HIF2α and VHL at the mRNA and protein levels (10). Nox4-dependent expression of HIF2α protein has been confirmed by others (14, 15).

Thus, HIF2α 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 HIF2α 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 DMEM supplemented with 10% FBS, penicillin, and streptomycin. 786-0 (WT) and 786-0 or empty pRC vector, respectively, were a gift from W. Kaelin, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA (16). They were selected with G418 (500 μg/mL) and recombinant phosphate buffer with 1 mmol/L ethylene glycol tetraacetic acid, 150 mmol/L sucrose, and 100 μmol/L NADPH. Lucigenin was added, and chemiluminescence read every 30 seconds for 20 minutes (SpectraMax Plus 384) and expressed as relative light units (RLU)/mg protein. Alternatively, ROSs 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).

Branching and invasion assays

Branching morphogenesis assays were performed as previously described (22). Briefly, 60-μL cells at 3.5 × 105 cells/mL media were mixed 1:1 with Matrigel in six flat-bottomed 96-well plates. After 30 minutes at 37°C, 120-μL DMEM with 10% FBS and 40 ng recombinant hepatocyte growth factor (HGF; Sigma-Aldrich) was added, and cells were incubated an additional 72 hours before determining the percentage of branched cells per well. Invasion assays were done in 8-μm Matrigel 24-well invasion chambers (BD). In the top chambers, 1.5 × 105 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.). Stained cells were photographed at >100 magnification and counted. Significance was determined by the Student t-test.

Immunofluorescence and confocal microscopy

A total of 5 × 105 cells were plated on cover-glass in 24-well plates for 12 hours. They were then incubated 4 hours with indicated media at 21% O2 or 1% O2. Cells were then fixed in 4% paraformaldehyde for 15 minutes, washed three times with PBS, then permeabilized with 0.1% Triton X-100 in PBS solution for 15 minutes. Cover slides were blocked with 2% BSA for
45 minutes and stained by primary antibodies for 60 minutes. After five washes with 0.5% BSA, secondary antibody was applied for 60 minutes, 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) 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 ×40 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). Tumors were measured twice weekly with digital calipers (VWR) 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 before 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-HIF2α 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 of <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 coactivators 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, whereas Caki1 cells express wild-type VHL. By semiquantitative RT-PCR, all three lines abundantly expressed Nox4 and the cofactor 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 (Supplementary Fig. S1).

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 (Supplementary Fig. S3). In summary, Nox4 was the dominant NADPH oxidase in three RCC cell 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 HIF2α 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 HGF and will migrate through a Matrigel-coated filter membrane toward an hepatocyte growth factor/scatter factor gradient. These behaviors are believed to reflect invasive and metastatic potential and are completely abrogated by reintroduction 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 (Supplementary Fig. S2A and S2B). 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 (0.125–10 mmol/L) 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 with 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 cotransduction 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). Cotransduction with Ad-catalase further decreased invasion in both cell lines (P < 0.01).

By 2',7'-dichlorofluorescein (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 manganese superoxide dismutase (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 with 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 TEMPOL was confirmed using a lucigenin chemiluminescent assay for NADP(H)-dependent superoxide generation from isolated cell membranes (Fig. 4F).

To summarize, scavenging of intracellular superoxide by either TEMPOL 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**

DTT is a strong antioxidant. However, under physiologic 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 (Supplementary Fig. S4). The number of invasive cells was decreased in a dose-dependent fashion, likely due to overall cell attrition. Despite this, we observed a nearly 2-fold increase in the percentage of 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 HIF2α**

We have reported that expression of HIF2α at the mRNA and protein level is suppressed by Nox4 silencing (10). Others have confirmed that HIF2α protein expression is dependent upon Nox4 expression (14, 15). However, we have observed suppression in HIF2α transactivation even at high HIF2α protein levels with Nox4 silencing. To test our hypothesis that Nox4 further contributes to activation of HIF2α, we used confocal microscopy to examine HIF2α cellular localization in our Nox4-silenced 786-0 and RCC4 cells. Activation is a multistep process that requires binding to the aryl hydrocarbon receptor nuclear translocator, nuclear translocation, binding to CBP/p300 and other cofactors, and binding to promoter DNA. Under hypoxic conditions, this requires factor-inhibiting HIF (FIH)-mediated hydroxylation of asparagine residues. As HIF2α has been shown to be less dependent upon FIH than HIF1α for activation (26), we hypothesize that Nox4 provides an alternate activating signal for HIF2α.

Figure 5A shows representative confocal images of HIF2α localization. We observed a Nox4-dependent distribution of endogenous HIF2α 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 HIF2α. However, Nox4 KD cells showed a striking nuclear exclusion of HIF2α with granular perinuclear enhancement. Notably, this nuclear...
exclusion was seen under both normal oxygen (Supplementary Fig. S5A–S5D) and hypoxic conditions, suggesting that for HIF2α, there may be a superoxide requirement even for hypoxic activation. Consistent with superoxide as a signaling intermediary, pretreatment with TEMPOL or transduction with Ad-SOD in NS cells mimicked the HIF2α 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 HIF2α expression following TEMPOL treatment, whereas nuclear HIF2α expression increased with DTT treatment (Fig. 5B and C). These findings support a role for Nox4-generated superoxide in nuclear accumulation of HIF2α.

**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 by week 5, whereas Nox4 knockdown (KD) tumors were not significantly smaller at 14 weeks than NS (mean cross sectional estimation of tumor inhibition. Consistent with this, immunohistochemical evaluation of the 14-week tumor explants revealed that protein expression of Nox4 and HIF2α was as high in the KD tumors as control (Supplementary Fig. S6c). 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 HIFα protein and increased expression of HIF transcription targets, occurs commonly in sporadic clear cell RCC. We previously reported that expression and transactivation of HIF2α 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 HIF2α under both normal and hypoxic oxygen conditions demonstrating that Nox4 is an alternative activating signal for HIF2α 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.

HIF1α and HIF2α 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 HIF2α predominance promotes cell survival, whereas HIF1α predominance favors apoptosis (27). In kidney cancer, HIFα expression is biased toward HIF2α, and inhibition of HIF2α suppresses VHL+/− tumor growth (6, 28). We speculate that heightened HIF2α transcriptional activity is due in part...
to constitutive, isofrom-specific induction by highly expressed renal Nox4. In renal cells, HIF2α 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 reexpression 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. A recent report by Boudreau and colleagues 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 and colleagues 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, a nonspecific flavoprotein inhibitor of Nox enzymes, and Nox4 silencing (31). Nox4 has been implicated in the regulation of cell migration in nonmalignant 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, ROSs have been shown to play a role in the tumorigenic phenotype of VHL-deficient, human RCC cells and that specific Nox4 suppression inhibits intracellular superoxide generation, prevents nuclear accumulation of HIF2α, and phenocopies reexpression 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 HIF2α. 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.

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

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