Reduced Expression of Fumarate Hydratase in Clear Cell Renal Cancer Mediates HIF-2α Accumulation and Promotes Migration and Invasion

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Abstract

Germline mutations of FH, the gene that encodes for the tricarboxylic acid TCA cycle enzyme fumarate hydratase, are associated with an inherited form of cancer referred to as Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC). Individuals with HLRCC are predisposed to the development of highly malignant and lethal renal cell carcinoma (RCC). The mechanisms of tumorigenesis proposed have largely focused on the biochemical consequences of loss of FH enzymatic activity. While loss of the tumor suppressor gene von Hippel Lindau (VHL) is thought to be an initiating event for the majority of RCCs, a role for FH in sporadic renal cancer has not been explored. Here we report that FH mRNA and protein expression are reduced in clear cell renal cancer, the most common histologic variant of kidney cancer. Moreover, we demonstrate that reduced FH leads to the accumulation of hypoxia inducible factor-2α (HIF-2α), a transcription factor known to promote renal carcinogenesis. Finally, we demonstrate that overexpression of FH in renal cancer cells inhibits cellular migration and invasion. These data provide novel insights into the tumor suppressor functions of FH in sporadic kidney cancer.

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Introduction

In 2010, over 57,000 men and women will be diagnosed with renal cell carcinoma (RCC) and 13,000 individuals will die of this disease [1]. Although survival for patients with localized disease is high, patients with advanced disease face a poor prognosis despite recent advances in targeted therapy (recently reviewed [10]). While loss of the von Hippel Lindau (VHL) tumor suppressor gene is thought to be an initiating event for the majority of RCCs [2], little is known about subsequent genetic events and their respective impact on tumorigenesis. Elucidation of these pathways will identify novel therapeutic targets as well as facilitate biomarker development that may have both diagnostic and prognostic significance.

Germline mutations of FH are associated with an inherited form of renal cancer referred to as Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC) [3,4]. FH encodes the tricarboxylic acid cycle enzyme fumarate hydratase (also referred to as fumarase) which catalyzes the hydration of fumarate to form malate. Individuals with HLRCC are predisposed to the development of leiomyomas of the skin and uterus in addition to highly malignant and lethal RCC. The mechanisms of tumorigenesis proposed have largely focused on the biochemical consequences of loss of FH enzymatic activity. It has been proposed that loss of FH leads to fumarate accumulation and promotes a pseudohypoxic state in which hypoxia response pathways are aberrantly activated despite normoxic conditions [5]. Fumarate has been shown to inhibit proline hydroxylation of the hypoxia inducible factors HIF-1α and HIF-2α which are catalyzed by a family of enzymes referred to as the HIF prolyl hydroxylases (PHDs) [5]. In their unhydroxylated form, HIFs avoid recognition by the E3 ubiquitin ligase VHL (which targets these proteins for proteosomal degradation) and are thus stabilized [6,7,8,9]. Under these conditions either HIF-1α or HIF-2α are able to heterodimerize with the constitutively expressed protein HIF-1β, also referred to as ARNT [recently reviewed [10]]. This heterocomplex is able to transcriptionally activate several genes including VEGF and other growth factors that may be pro-tumorigenic when dysregulated. Pseudohypoxia has also been implicated in the most common variant of RCC, clear cell carcinoma (ccRCC), in which loss of VHL is a common genetic
event [11]. As expected, elevated levels of HIF-1α and/or HIF-2α are noted in clear cell renal cancers [12,13]. Interestingly, several lines of evidence indicate that HIF-2α as opposed to HIF-1α, is critical to RCC formation and/or progression [14,15,16].

While VHL loss is clearly critical to HIF-2α stabilization, alternate mechanisms, besides the prevention of degradation, may play a role in the maintenance of HIF-2α in renal cancer. Previous work by Block et al. established a role for reactive oxygen species (ROS) generated by NADPH oxidases in maintaining HIF-2α protein expression through an AKT-dependent mRNA translational mechanism in VHL-deficient cells [17]. In addition, mTOR signaling complex 2 (mTORC2), a known activator of AKT signaling, has been shown to promote HIF-2α accumulation in VHL null renal carcinoma cells [18]. More recently, treatment of RCC cells with a dual PI3K/mTOR inhibitor suppressed the expression of HIF-2α [19]. These data support the notion that ongoing HIF-2α synthesis is critical to the maintenance of this oncogenic transcription factor in renal cancer cells.

FH mutations have primarily been linked to papillary type II renal cancer, a histologic variant that accounts for less than 10% of all renal cancers [20]. FH mutations have not been identified in sporadic clear cell renal cancer. However, a recent report has linked FH to the development of clear cell renal cancer in a patient with a germline mutation of FH [21]. To date, the expression and function of FH in ccRCC has not examined. Therefore, we investigated the role of FH in sporadic clear cell renal cancer.

### Results

**Reduced expression of FH in clear cell renal carcinoma**

FH expression in ccRCC has yet to be explored. Therefore, we first examined the protein expression of FH in a panel of human clear cell renal tumors and patient-matched normal renal parenchyma. Immunoblot analysis of tissue lysates demonstrated a marked reduction of FH protein levels in the tumors as compared to normal adjacent tissue (Figure 1A). To confirm our findings, we performed immunohistochemical staining for FH on patient matched tumor/normal pairs. These results correlated with our immunoblotting results in that staining for FH was less in tumors relative to control renal tissue (Figure 1B). We next examined FH protein levels in a panel of established ccRCC cell lines (786-O, A498, RCC4, and ACHN). All cultured cell lines demonstrated reduced FH protein levels relative to normal kidney (Figure 1C). We then examined mRNA expression levels of FH in tumor tissue as compared to patient-matched normal renal tissue in specimens from the Cooperative Human Tissue Network (NCI). Quantitative real time RT-PCR demonstrated reduced FH mRNA levels in tumor tissue as compared with normal tissue (Figure 2). Analysis of mRNA levels reveals that over 70% of the tumor samples demonstrated reduced FH mRNA levels relative to normal matched renal parenchyma. Moreover, 15/32 patient samples (47%) demonstrated a greater than twofold reduction in FH mRNA levels in tumor samples relative to normal control. Overall, the average reduction in FH mRNA levels was 2.9 fold. This difference was determined to be statistically significant. These

![Figure 1. FH protein expression is reduced in clear cell renal cancer.](https://example.com/fh.png)
results demonstrate reduced expression of FH at the mRNA and protein levels in ccRCC.

**FH modulates HIF-2α levels**

High levels of fumarate in FH-deficient RCC play a role in stabilizing HIF-2α protein expression through inhibition of proline hydroxylase activity thereby preventing VHL recognition. Based on these data, we hypothesized that loss of FH should have no impact on HIF protein levels in VHL null cell lines. However, we found that siRNA-mediated knockdown of FH resulted in a further increase of HIF-2α protein levels in two ccRCC lines which are VHL null (786-O and A498) (Figure 3A). In support of these findings, transient overexpression of FLAG-tagged FH (FH-FLAG) reduced HIF-2α levels in 786-O cells (Figure 3B). Together, this suggests that FH modulates HIF-2α protein expression in the absence of VHL.

To further elucidate the mechanism by which FH regulate HIF-2α protein expression, we created stable cells lines overexpressing FH-FLAG in VHL-deficient 786-O cells. We identified 3 clones that stably expressed the FH-FLAG construct. All 3 clones demonstrated reduced HIF-2α levels as compared with control vector transfected cells (Figure 3C). We initially considered whether the effects on HIF-2α were transcriptionally mediated, however we did not detect reductions in HIF-2α mRNA levels with FH overexpression (data not shown).

**FH loss activates AKT signaling**

Recent reports have implicated PI3K/AKT signaling in maintaining HIF-2α protein expression in VHL null cells through a translational mechanism [17,18,19,22]. We examined AKT signaling with FH modulation. We found that siRNA-mediated knockdown of FH in both 786-O and A498 cells results in increased AKT phosphorylation on serine 473 of AKT (Figure 4A). Correspondingly, overexpression of FH lowered phospho-AKT levels in 786-O cells (Figure 4B) indicating that FH levels inversely correlate with AKT signaling. We next

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**Figure 2. FH mRNA expression is reduced in clear cell kidney cancer.** mRNA levels of FH were determined in a separate set of tumor samples relative to patient matched normal renal parenchyma with real time RT-PCR (p = 0.004). Expression levels were normalized to 18 s rRNA levels prior to comparative analysis. doi:10.1371/journal.pone.0021037.g002

**Figure 3. FH expression impacts HIF-2α levels.** A) VHL null 786-O and A498 cells were transected with siRNA to FH and scramble control (siNC). Forty-eight hours following transfection, protein lysates were analyzed by immunoblotting for the indicated proteins. B) 786-O cells were transiently transfected with control vector (CV) and vector containing FLAG tagged FH. Forty-eight hours following transfection, protein lysates were analyzed by immunoblotting for the indicated proteins. FLAG immunoblot indicates successful expression of the transgene. C) (Left) 786-O cells were stably transfected with CV and FH-FLAG. After selection in puromycin, single cell clones were harvested and screened for FLAG expression. HIF-2α levels were measured in FH-FLAG expressing clones relative to CV transfected cells. Densitometry of the bands is quantitatively displayed on the right. Mean relative values +/- standard deviation were obtained with ImageJ software from independent experiments. doi:10.1371/journal.pone.0021037.g003
examined the effects of PI3K inhibition on FH-dependent HIF-2α protein expression. Consistent with our previous findings, FH knockdown activated AKT signaling and increased HIF-2α levels compared to scramble transfected cells (siNC) (Figure 4C). However, cotreatment of transfected cells with the PI3K inhibitor LY-294002 blocked the increase in HIF-2α associated with FH knockdown (Figure 4C). Together, this suggests that FH maintains HIF-2α protein expression through mechanisms dependent on PI3K and AKT signaling.

FH expression mediates cell migration and invasion

The biological consequences of FH overexpression in RCC cells were next examined. FH null tumors are highly invasive and often metastatic tumors [20], and HIF-2α has previously been implicated in this cellular process [23]. Therefore, we investigated the role of FH in cellular migration and invasion in ccRCC. We find that knockdown of HIF-2α with siRNA in 786-O RCC cells diminished cellular motility as determined by wound healing assay compared to scramble transfected cells (Figures 5A and 5B). Quantification of these results are provided in Figure 5C. While almost 80% of the wound gap was closed in control transfected cells by 12 hours, 50% of the wound gap remained in HIF-2α knockdown cells. Given these data, we examined wound healing in FH overexpressing 786-O subclones as compared with control vector transfected cells. Both FH overexpressing clones had significantly reduced wound closure as compared with control vector transfected cells, suggesting that loss of FH contributes to migration in RCC (Figure 6A). In control vector transfected cells, the wound width gap was almost completely closed by 10 hours. In contrast, FH overexpressing cells closed the wound gap by only half by 10 hours indicating reduced cellular migration was a result of FH overexpression. These results are displayed graphically (Figure 6B). To further corroborate these data, we examined cellular migration utilizing a chamber assay with 10% fetal bovine serum as the chemottractant. 786-O vector control cells were significantly more migratory than FH overexpressing clones (Figure 6C). Finally, overexpression of FH in RCC cells reduced their invasive ability as determined by matrigel invasion assay (Figure 6D). Taken together, these data demonstrate that loss of FH expression enhances the migratory and invasive ability of RCC cells.

Discussion

In this report, we demonstrate for the first time reduced FH expression at the mRNA and protein levels in clear cell renal carcinoma, the most common histological variant that accounts for the majority of kidney cancers. These findings are significant as previous studies did not identify FH mutations in RCC lines as well as primary RCC specimens [24]. The mechanisms by which mRNA levels of FH are reduced are under current investigation. Hypermethylation may be one mechanism by which FH expression is suppressed. Dulaimi et al. did not identify hypermethylation in a CpG island of the FH promoter in a panel of papillary RCCs [25]. However, no studies to date have examined FH methylation status in ccRCC. While hypermethylation is a means of tumor suppressor gene silencing, alternate mechanisms may also account for the reduced expression of FH we have identified. Recent interest has focused on the role of...
microRNA (miRNA) in gene regulation where reports suggest that genes involved in oxidative phosphorylation may be subject to regulation by miRNAs [26]. Clearly, these possibilities warrant further investigation given the biological significance of our findings.

We have previously demonstrated that reintroduction of wild type FH into a FH deficient tumor cell line results in a marked reduction in nuclear HIF-1α levels [27]. Moreover, siRNA mediated knockdown of FH has been shown to increase HIF-1α levels in A549 lung carcinoma cells which express VHL [5]. The findings from these studies, as well as other biochemical studies, suggest that the principle mechanism by which this occurs is through HIF protein stabilization via inhibition of prolyl hydroxylase activity as a result of FH loss. These findings would therefore presume that the effect of FH on HIF would only be evident in the presence of VHL. However, our findings are quite novel in that they indicate that FH can modulate HIF-2α independently of VHL. VHL-independent pathways that mediate HIF degradation have been reported. In particular, Hsp90 and RACK1 have previously been shown to modulate HIF-1α degradation [28]. Therefore, it is possible that a similar mechanism mediates HIF-2α degradation as well. Despite stabilization via inhibition of protein degradation, there is growing evidence that alternate pathways play a role in maintaining HIF-2α protein levels in the absence of VHL. Block et al. demonstrated that elevated cellular reactive oxygen species, mediated by p22-phox based Nox oxidases, maintain HIF-2α protein levels in RCC cells through an AKT/4E-BP1 mRNA translational dependent mechanism [17,22]. Correspondingly, phosphorylation of AKT and 4E-BP1 are enhanced in human RCC tissue relative to normal parenchymal tissue [22]. More recently, Toschi et al. found that AKT activation, via signaling through the mTOR signaling complex 2 (mTORC2), was required to maintain HIF-2α in VHL null cells [18]. AKT activation is a common signaling node in cancer and alternate mechanisms may lead to AKT activation in RCC including loss of FH.

The mechanism by which AKT signaling is activated by FH loss is under current investigation. We have previously demonstrated that loss of FH in renal epithelial cells results in elevated cellular oxidative stress [27]. Hence, ROS may be a contributor to the generation of HIF-2α...

Figure 5. HIF-2α promotes migration in RCC cells. 786-O RCC cells were transfected with scramble control siRNA (siNC) or siRNA to HIF-2α. Western blotting results of whole cell extracts are demonstrated in panel A. B) Wound healing assay was performed in transfected cells. Images were serially taken at the indicated time point following "wound" induction. Results are graphically displayed in panel C. Asterisks (*) indicate statistical significance with p<0.05 relative to scramble control transfected cells. doi:10.1371/journal.pone.0021037.g005
elevated HIF-2α levels upon FH knockdown. Alternatively, the effects of FH loss may be unrelated to its role in the TCA cycle. It is well established that FH also exists in an extramitochondrial, cytosolic form. At this time, very little is known about the function of this form of FH. However, recent evidence provided by O’Flaherty et al. indicates that loss of extramitochondrial FH may contribute to HIF stabilization [29]. In addition, cytosolic FH has been implicated in the DNA damage response [30]. Upon DNA damage, cytosolic FH has been shown to translocate into the nucleus. The mechanism by which FH participates in the DNA damage response remains unclear. However, there is certainly the possibility that FH and the metabolites it interacts with may regulate the function of other proteins, potentially within the nucleus. Interestingly, AKT has also been shown to function in the nucleus [31]. Given our data, as well as these recent reports, targeting AKT mediated signaling pathways, either at the level of AKT or upstream, may prove to be of therapeutic benefit for renal cancer. This is in concordance with recent data demonstrating the in vitro and in vivo efficacy of a dual PI3K/mTOR inhibitor in RCC [19].

Interestingly, there is precedent for alterations of TCA cycle enzyme in cancer. Multiple other genes encoding enzymes of the tricarboxylic (TCA) cycle are considered tumor suppressor genes including SDHB, SDHC, and SDHD (Succinate Dehydrogenase subunits B,C,D) [32,33,34]. SDH subunit mutations have been linked to pheochromocytoma and paraganglioma and more recently to gastrointestinal stromal tumor (GIST) [35]. In addition to mutations, alterations of expression of these genes have been linked to malignancy. Dahia et al. demonstrated reduced expression of succinate dehydrogenase subunit B (SDHB) in a subset of pheochromocytomas [36]. More recently, reduced expression of SDHB was identified in large proportion of GISTs without mutations in SDHB or other genes commonly mutated in GISTs including KIT and PDGFRA [35]. Based on these data, a potential unifying theme may be that defects in oxidative phosphorylation, either through mutation or expression changes, have a role in oncogenesis. Recently, Chen et al. proposed that oxygen consumption via mitochondrial metabolism may regulate tumor growth by limiting the availability of oxygen for nonmitochondrial activities that are contributory to tumor growth [37].

Of significant interest is our finding that FH overexpression results in reduced migration and invasion of RCC cells. Our data are in concordance with a recent report by Costa et al. that demonstrated that fh knockdown in immortalized mouse embryonic fibroblasts (iMEFs) resulted in increased motility as compared with untransduced iMEFS [38]. Moreover, the increase in motility was HIF-1α dependent. The role of HIF-2α in their studies could not be determined as they were unable to detect HIF-2α in their studies. In our studies, we found that HIF-2α expression in fh deficient iMEFS. Our studies focused on HIF-2α given prior studies that implicate its role in renal carcinogenesis. Given that HIF-2α knockdown in RCC cells inhibits migration, our data indicate that the effects of FH overexpression on migration and invasion are, in part, mediated by effects on HIF-2α. HIF-2α has previously been implicated in the invasive behavior of RCC cells. Moreover the invasive promoting properties of HIF-2α have been studied in 786-O cells, the same cells utilized in this study. Hughes et al. demonstrated that HIF-2α knockdown in 786-O cells reduced the expression of multiple integrins that may mediate cell motility and invasion [39]. Petrella et al. examined the role of VHL loss in cell invasion [40]. They found that reintroduction of wild type VHL into VHL-deficient 786-O cells reduced HIF-2α levels and cell invasion. Conversely, re-expression of HIF-2α in VHL reconstituted 786-O cells restored invasive potential. These data indicate a role for HIF-2α in RCC migration and invasion. Additionally, HIF-2α overexpression was found to enhance the growth of RCC xenografts whereas overexpression of HIF-1α was found to inhibit xenograft growth [41]. Correspondingly, separate studies indicate that HIF-2α, but not HIF-1α, contributes to the growth of VHL null tumor xenografts [16,42]. Hence, our data add to the growing body of evidence demonstrating the tumor-promoting effects of HIF-2α expression in RCC.

Despite the recent approval of multiple agents for advanced renal cancer, most patients with advanced renal cancer will eventually succumb to their disease. Hence, the identification of novel signaling pathways will be critical to the development of effective therapeutics. There is now mounting evidence that renal cancer is among the tumors that are representative of the emerging paradigm in cancer biology of metabolic links to malignancy. Our findings with FH suggest a metabolic reprogramming in clear cell renal cancer that promotes expression of tumorigenic factors including HIF-2α via multiple mechanisms. Unraveling the mechanisms by which tumor metabolism is altered and the downstream cellular consequences should provide deep insight in renal cancer biology as well as novel therapeutic strategies.

Materials and Methods

Cells

A498, 786-O, and ACHN cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified 5% CO2 atmosphere. RCC4 cells were kindly provided by P. Ratcliffe (Oxford).

Chemicals

LY294002 was purchased from Sigma.

Constructs

The FH-FLAG construct has been previously described [27].

Immunoblotting

All immunoblot analyses were performed as previously described [27] on whole-cell lysates prepared with the use of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) supplemented with protease inhibitor cocktail (Roche). Antibodies were obtained from the following commercial sources: GeneTex (FH-immunoblotting), Santa Cruz Biotechnology (FH-immunohistochemistry), Novus (GAPDH, HIF-2α), Sigma (Tubulin, β-Actin), Cell Signaling (total AKT and ser473 phospho AKT).
Tissue sample quantitative real-time PCR

Biopspecimens for RNA analysis were obtained from the Cooperative Human Tissue Network of the NCI/NIH. Total RNA was reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems). cDNA was then used as template with Applied Biosystems’ assays-on-demand 20 x assay mix of primers and Taqman probes. Forty amplification cycles were done on the Applied Biosystems Prism 7900 sequence detector. Fold change values between tumor and normal samples were calculated using the ΔΔCt method with normalization to 18S rRNA levels. As a statistical test we used the Mann-Whitney paired non-parametric test to compare FH expression in tumor versus normal adjacent tissue (implemented in GeneSpring, Agilent).

Immunoblotting and immunohistochemistry of human tumor specimens

Tumor samples and normal corresponding tissue from patients with RCC were obtained from the Department of Urology at the University of Texas Health Science Center at San Antonio. The tumors for this study were histologically classified as clear cell renal carcinoma by a genitourinary pathologist. The collection and handling of human samples was performed according to a protocol approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio. Based on the protocol, samples were obtained in a deidentified fashion from patients undergoing surgical resection for renal cancer. As samples were obtained and analyzed in an anonymous fashion, patient consent was not required.

Wound healing assay

Cells were allowed to grow to near confluence in 60 mm dishes. A uniform scratch was then made down the center of the plate using a 200 microliter micropipette tip, followed by washing twice with PBS. The same marked field of the scratch wound was photographed using an Olympus light microscope (4× objective) at the indicated time points. The width of the scratch wound was measured at three different areas with Q-Capture pro software. Quantified data represent the mean +/− S.D. from at least two independent experiments.

Migration assay

786-O subclones cells (1×10⁵) were seeded on an 8 μm pore size Thin-cert for 24 well plates (Greiner Bio-One) in serum free media. Seven hundred fifty microliters of 10% FBS medium was added to the bottom chamber as chemotractant. After 48 hrs, cells on the top of the membrane were removed with a cotton swab. The migrated cells at the bottom side were washed with PBS, fixed with 70% ethanol and stained using 0.1% Crystal violet to visualize the migrated cells. Migrated cells attached to the lower side of the membrane were enumerated using a light microscope at 10× magnification. Counts represent the average cell number of ten microscopic fields.

Invasion assay

Cell invasion was determined by invasion assay (membrane coated with a layer of Matrigel extracellular matrix proteins) according to the manufacturer’s instructions. Cells were seeded in serum-free medium into the upper chamber and invaded toward the bottom chamber containing a 10% FBS medium as the chemotractant. Membranes were processed in a similar fashion as the migration assay.

RNA interference

For FH and HIF-2α knockdown, cells were transfected with pooled siRNA reagent (Thermo Fisher) with the Amaza Nucleofector system according to the manufacturer’s protocol. Cells were harvested at 48–72 hours following transfection. A non-targeting scramble siRNA pool was used as a negative control (Thermo Fisher).

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Author Contributions

Conceived and designed the experiments: SS SLN LZS KB. Performed the experiments: SS KS SL IY CFO. Analyzed the data: SS CBL LZS KB. Contributed reagents/materials/analysis tools: DJP. Wrote the paper: SS KB.

References

1. Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. CA Cancer J Clin 60: 27–90.
2. Gnarra JR, Tory K, Weng Y, Schmidt L, Wei MH, et al. (1994) Mutations of the VHL tumour suppressor gene in renal carcinoma. Nat Genet 7: 83–90.
3. Launonen V, Vierimaa O, Kiuru M, Isola J, Roth S, et al. (2001) Inherited susceptibility to uterine leiomyomas and renal cell cancer. Proc Natl Acad Sci U S A 98: 3387–3392.
4. Tomlinson IP, Alam NA, Rowan AJ, Barclay E, Jaeger EE, et al. (2002) Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nat Genet 30: 406–410.
5. Isaacs JS, Jung YJ, Mole DR, Lee S, Torres-Cabala C, et al. (2005) HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. Cancer Cell 8: 143–153.
6. Epstein AC, Gleadle JM, McNell LS, Hewison KM, O’Rourke J, et al. (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107: 43–54.
7. Ivan M, Kondo K, Yang H, Kim W, Valliand J, et al. (2001) HIFαAlpha targeted for VHL-mediated degradation by proline hydroxylation: implications for O2 sensing. Science 292: 464–468.
8. Jakkola P, Mole DR, Tian YM, Wilson MJ, Giebert J, et al. (2001) Targeting of HIF-α for the von Hippel-Lindau ubiquitination complex by O2-regulated prolyl hydroxylation. Science 292: 468–472.
9. Brückl RK, M Knigt SL (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294: 1337–1340.
10. Gordan JD, Simon MC (2007) Hypoxia-inducible factors: central regulators of the tumor phenotype. Curr Opin Genet Dev 17: 71–77.
11. Bratslavsky G, Sudarshan S, Necker L, Linehan WM (2007) Pseudohypoxic pathways in renal cell carcinoma. Clin Cancer Res 13: 4667–4671.
12. Wiesner MS, Munchenbach PM, Berger I, Morgan NV, Roigas J, et al. (2001) Constitutive activation of hypoxia-inducible genes related to overexpression of hypoxia-inducible factor-1alpha in clear cell renal carcinomas. Cancer Res 61: 5213–5222.
13. Zhang N, Gong K, Yang XY, Xin DQ, Na YQ (2006) [Expression of hypoxia-inducible factor-1alpha, hypoxia-inducible factor-2alpha and vascular endothelial growth factor in sporadic clear cell renal cell renal cell carcinoma and their significance in the pathogenesis thereof]. Zhonghua Yi Xue Za Zhi 86: 1526–1529.
14. Riepsools O, Kibel A, Gray S, Kaelin WG, Jr. (1995) Tumour suppression by the human von Hippel-Lindau gene product. Nat Med 1: 822–826.
15. Kondo K, Kloc J, Nakamura E, Lechpammer M, Kaelin WG, Jr. (2002) Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell 1: 237–246.
16. Maraniche JK, Vassell JR, Rios J, Bonafacino JS, Linehan WM, et al. (2002) The contribution of VHL substrate binding and HIF-1alpha to the phenotype of VHL loss in renal cell carcinoma. Cancer Cell 1: 247–253.
17. Block R, Gorin Y, Howver P, Williams P, Gielbert M, et al. (2007) NADPH oxidases regulate HIF-2alpha protein expression. J Biol Chem 282: 8019–8026.
18. Toschi A, Lee E, Gadir N, Ohh M, Foster DA (2008) Differential dependence of hypoxia-inducible factors 1 alpha and 2 alpha on mTORC1 and mTORC2. J Biol Chem 283: 54495–54499.
19. Cho DC, Cohen MB, Panka DJ, Collin M, Giebertsmichael M, et al. (2010) The efficacy of the novel dual PI3-kinase/mTOR inhibitor NVP-BEZ235 compared with rapamycin in renal cell carcinoma. Clin Cancer Res 16: 3626–3636.
20. Grubb RL, 3rd, Franks ME, Toro J, Middelton L, Choyke L, et al. (2007) Hereditary leiomyomatosis and renal cell cancer: a syndrome associated with an aggressive form of inherited renal cancer. J Urol 177: 2074–2079; discussion 2079–2080.

21. Lehtonen HJ, Blanco I, Puolats JM, Herva R, Launonen V, et al. (2007) Conventional renal cancer in a patient with fumarate hydratase mutation. Hum Pathol 38: 793–796.

22. Block K, Gorin Y, New DD, Eid A, Chehnicki T, et al. (2010) The NADPH oxidase subunit p22phox inhibits the function of the tumor suppressor protein tuberin. The American journal of pathology 176: 2447–2455.

23. Kim WV, Pererra S, Zhou B, Carretero J, Yeh JJ, et al. (2009) HIF2alpha cooperates with RAS to promote lung tumorigenesis in mice. The Journal of clinical investigation 119: 2160–2170.

24. Block K, Gorin Y, New DD, Eid A, Chelmicki T, et al. (2010) The NADPH oxidase subunit p22phox inhibits the function of the tumor suppressor protein tuberin. The American journal of pathology 176: 2447–2455.

25. Morris MR, Maina E, Morgan NV, Gende D, Astuti D, et al. (2004) Molecular genetic analysis of FH1, FH, and SDHB candidate tumour suppressor genes in renal cell carcinoma. J Clin Pathol 57: 706–711.

26. Dulaimi E, Ibanez de Caceres I, Uzzo RG, Al-Saleem T, Greenberg RE, et al. (2004) Promoter hypermethylation profile of kidney cancer. Clin Cancer Res 10: 3972–3979.

27. Chen Z, Li Y, Zhang H, Huang P, Luthra R (2010) Hypoxia-regulated microRNA-210 modulates mitochondrial function and decreases ISCU and COX10 expression. Oncogene 29: 4362–4368.

28. Sudarshan S, Sourbier C, Kong HS, Block K, Valera Romero VA, et al. (2009) Fumarate hydratase deficiency in renal cancer induces glycolytic addiction and hypoxia-inducible transcription factor 1alpha stabilization by glucose-dependent generation of reactive oxygen species. Mol Cell Biol 29: 4080–4090.

29. Liu YY, Back JH, Zhang H, Diez R, Cole RN, et al. (2007) RACK1 competes with HSP90 for binding to HIF-1alpha and is required for O2-dependent and HSP90 inhibitor-induced degradation of HIF-1alpha. Molecular cell 25: 207–217.

30. O’Flaherty L, Adam J, Heather LC, Zhdanov AV, Chung YL, et al. (2010) Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. Proc Natl Acad Sci U S A.

31. Wang R, Brattain MG (2006) AKT can be activated in the nucleus. Cell Signal 18: 1722–1731.

32. Astuti D, Lafit F, Dallol A, Dahia PL, Douglas F, et al. (2001) Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. Am J Hum Genet 69: 49–54.

33. Baysal BE, Ferrell RE, Willet-Brozick JE, Lawrence EG, Myszorek D, et al. (2000) Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. Science 287: 649–651.

34. Niemann S, Muller U (2000) Mutations in SDHC cause autosomal dominant paraganglioma, type 3. Nat Genet 26: 268–270.

35. Janeway KA, Kim SY, Lodish M, Nose V, Rustin P, et al. (2010) Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. Proc Natl Acad Sci U S A.

36. Dahia PL, Ross KN, Wright ME, Hayashida CY, Santagata S, et al. (2005) A HIF1alpha regulatory loop links hypoxia and mitochondrial signals in pheochromocytomas. PLoS Genet 1: 72–80.

37. Chen Y, Cairns R, Papandreses I, Koong A, Denko NC (2009) Oxygen consumption can regulate the growth of tumors, a new perspective on the Warburg effect. PLoS One 4: e7033.

38. Costa B, Detori D, Lorenzato A, Bardella C, Cothella N, et al. (2010) Fumarase tumor suppressor gene and MET oncogene cooperate in upholding transformation and tumorigenesis. FASEB J 24: 2680–2688.

39. Hughes MD, Kapllani E, Alexander AE, Burk RD, Schoenfeld AR (2007) HIF-2alpha downregulation in the absence of functional VHL is not sufficient for renal cell differentiation. Cancer cell international 7: 13.

40. Petrella BL, Brinckerhoff CE (2006) Tumor cell invasion of von Hippel Lindau renal cell carcinoma cells is mediated by membrane type-1 matrix metalloproteinase. Molecular cancer 5: 66.

41. Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, et al. (2005) Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol Cell Biol 25: 5675–5686.

42. Kondo K, Kim WY, Lechpammer M, Kaelin WG, Jr. (2003) Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. PLoS Biol 1: E83.