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TITLE: Characterization of a Novel Critical Interplay Between VHL Inactivation and Iron Metabolism in Clear Cell Renal Cell Carcinoma Tumorigenesis

PRINCIPAL INVESTIGATOR: Eric C. Kauffman, MD

CONTRACTING ORGANIZATION: Roswell Park Comprehensive Cancer Center, Buffalo, NY

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Eric C. Kauffman, MD

E-Mail: eric.kauffman@roswellpark.org

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14. ABSTRACT This project investigates the interplay between cellular iron accumulation and von Hippel Lindau (*Vhl*) gene signaling dysregulation in clear cell renal cell carcinoma (ccRCC) tumorigenesis. The objective of the project is to introduce *Vhl* gene loss into the FeNTA mouse model of kidney cancer, and to perform other assays in human cells/tissues, in order to test the central hypothesis that cellular iron accumulation cooperates with Vhl loss to promote ccRCC tumorigenesis. Specific aims of this proposal are to: 1) determine how *Vhl* genetic loss alters the incidence, latency and histology of iron-induced mouse ccRCC tumorigenesis; 2) determine how *Vhl* genetic loss alters the genomic and immunologic landscape of iron-induced murine RCC tumors, including relative to the human ccRCC landscape; and 3) determine the role of the Vhl protein target, HIF-2α, in inducing iron dependency of Vhl-inactivated ccRCC cells. A key feature of this project is the novel introduction of *Vhl* loss into a carcinogenic animal model of RCC, which for the first time will allow *in vivo* study of molecular and cellular ccRCC phenotypes that are induced by *Vhl* loss, including genomic and immunologic effects. Current progress on Aim 1 includes completion of mouse treatments with iron, however longer follow up is needed to determine the effects on renal tumorigenesis. Work on Aim 2 will be performed in the latter half of the next year after development and harvest of murine tumors. Work on Aim 3 is mostly completed and suggests that iron-dependency of VHL-inactivated ccRCC cells is independent of HIF-2α overexpression. These findings collectively support a HIF-2α-independent synergistic interplay between VHL dysregulation and iron dysregulation in ccRCC.

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None listed.

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1. INTRODUCTION:

The subject of this project is the interplay between cellular iron accumulation and von Hippel Lindau (*Vhl*) gene signaling dysregulation in clear cell renal cell carcinoma (ccRCC) tumorigenesis. Vhl gene inactivation is critical but alone inadequate for ccRCC tumorigenesis, and cooperating alterations are largely unknown. A novel role for cellular iron accumulation in Vhl signaling dysregulation and ccRCC tumorigenesis has been suggested by observations from us and others, including the observation that iron accumulation in mouse kidneys in response to ferric iron nitrilotriacetic acid (FeNTA) treatment leads to tumors that mimic human ccRCC histologically, although the molecular and immunologic tumor biology is unknown. The objective of the current project is to introduce *Vhl* gene loss into the FeNTA mouse model and perform other assays in human cells/tissues to test the central hypothesis that cellular iron accumulation cooperates with Vhl loss to promote ccRCC tumorigenesis. Specific aims of this proposal are to 1) determine how *Vhl* genetic loss alters the incidence, latency and histology of iron-induced mouse ccRCC tumorigenesis; 2) determine how *Vhl* genetic loss alters the genomic and immunologic landscape of iron-induced murine RCC tumors, including relative to the human ccRCC landscape; and 3) determine the role of the Vhl protein target, HIF-2α, in inducing iron dependency in vhl-inactivated ccRCC cells. A key feature of this project is the novel introduction of genetically engineered *Vhl* loss into a carcinogenic animal model of RCC, which for the first time will allow *in vivo* study of molecular and cellular ccRCC phenotypes that are induced by *Vhl* loss, including genomic and immunologic effects. This combined genetic/carcinogenic model will establish a novel autochthonous mouse model of *Vhl*-inactivated ccRCC that may be genomically and immunologically similar to human ccRCC, which may provide an invaluable pre-clinical tool to investigate novel preventative and therapeutic strategies for this disease.

2. KEYWORDS:

- Clear cell (cc)
- Renal cell carcinoma (RCC)
- Autochthonous mouse model
- Iron
- Von Hippel Lindau (vhl)
- Hypoxia inducible factor 2 alpha (HIF-2α)
- PAX8
- Magnetic resonance imaging (MRI)

3. ACCOMPLISHMENTS:

What were the major goals of the project?

- Summary of Major Goals/Tasks, including expected and actual % completion:

Aim#.MajorGoal#	Proposed Months for Completion	Actual % Completion
1.1	**1-18**	**60%**
1.2	**18-24**	**0%**
1.3	**19-24**	**0%**
2.1	**19-24**	**0%**
2.2	**18-24**	**0%**
3.1	**1-18**	**90%**
3.2	**1-24**	**100%**

- Detailed breakdown list of Major Goals/Tasks and Subtasks, including actual % completion:

	Months Proposed to Complete	% Actual Complete
Specific Aim 1: Determine how vhl genetic loss alters the incidence, time-frame and histopathology of iron-induced murine ccRCC tumorigenesis.		
Aim 1, Major Task 1: Determine *Vhl* inactivation effect on iron-induced tumor burden using magnetic resonance imaging (MRI) T2-weighted relaxometry.	**1-18**	**60%**
Subtask 1: Complete generation of *vhl*$^{+/+}$ (n=50), *vhl*$^{+/-}$ (n=50), and *vhl*$^{-/-}$ (n=50) mouse cohorts by breeding *vhl*-floxed mice and heterozygous Pax8-Cre mice	1-3.5	70%
Subtask 2: Treatment of *vhl*$^{+/+}$, *vhl*$^{+/-}$ or *vhl*$^{-/-}$ mouse cohorts with intraperitoneal FeNTA	6-8	70%

injections for 12 weeks		
Milestone Achieved: Iron treatments of mouse cohorts complete	**8**	**70%**
Subtask 3: MRI of *vhl*$^{+/+}$, *vhl*$^{+/-}$ or *vhl*$^{-/-}$ mouse cohorts for time-point #1 (6 months post-treatment initiation)	12	0%
Subtask 4: MRI image analysis including tumor quantification and T2-weighted relaxometry measurement of tissue iron, for time point #1 (6 months post-treatment initiation)	12	0%
Milestone Achieved: **MRI analysis of mouse renal tumor burden at time point #1 (6 months post-treatment initiation) complete**	**12**	**0%**
Subtask 5: MRI of *vhl*$^{+/+}$, *vhl*$^{+/-}$ or *vhl*$^{-/-}$ mouse cohorts for time-point #2 (12 months post-treatment initiation)	18	0%
Subtask 6: MRI analysis including tumor quantification and T2-weighted relaxometry measurement of tissue iron, for time point 1 (12 months post-treatment initiation)	18	0%
Milestone Achieved: **MRI analysis of mouse renal tumor burden at time point #2 (12 months post-treatment initiation) complete**	**18**	**0%**
Aim 1, Major Task 2: Assess HIF-α dysregulation and iron accumulation in iron-induced mouse kidney tumors	**18-24**	**0%**
Subtask 1: Mouse kidney/tumor tissue harvest at 12 months post-treatment initiation	18	0%
Subtask 2: Mouse kidney/tumor tissue immunohistochemistry for HIF-α pathway members	19-21	0%
Subtask 3: Prussian Blue stain for total non-heme iron levels in mouse kidney/tumor tissues	19-21	0%
Subtask 4: Scoring and analysis of tissue staining levels for HIF-α pathway and iron	22-24	0%
Milestone Achieved: Measurement and analysis of HIF-α protein and iron levels in mouse kidney and tumor tissue	**24**	**0%**
Aim 1, Major Task 3: Histopathologic comparison among mouse cohorts.	**19-24**	**0%**
Subtask 1: Histopathologic determination of tumor incidence, number and size	19-20	0%
Subtask 2: Pathologist assessment of tumor histology, including nuclear grade, T stage, clear cell/papillary/eosinophilic features, necrosis and sarcomatoid differentiation	21-23	0%
Subtask 3: Comparison of tumor incidence, number, size, histology between mouse cohorts	24	0%
Milestone Achieved: **Comparison of tumor incidence, number, size and histology among *vhl*$^{+/+}$, *vhl*$^{+/-}$ or *vhl*$^{-/-}$ mouse cohorts**	**24**	**0%**
Specific Aim 2: Determine how VHL loss alters the genomic and immunologic landscape of iron-induced murine ccRCC tumors, including their similarity to human VHL-mutant ccRCC		
Aim 2, Major Task 1: Determine effect of *vhl* inactivation on the genomic profile of iron-induced murine ccRCC	**19-24**	**0%**

Subtask 1: Whole exome sequencing (WES) of mouse kidney/tumor tissue	19	0%
Subtask 2: RNAseq of mouse kidney/tumor tissue	19	0%
Subtask 3: WES and RNAseq bioinformatics analyses	20-24	0%
Milestone Achieved: **WES and RNAseq comparison of mouse kidney/tumor tissues among *vhl*$^{+/+}$, *vhl*$^{+/-}$ or *vhl*$^{-/-}$ mouse cohorts**	**24**	**0%**
Aim 2, Major Task 2: Determine effect of *vhl* loss on density and subtypes of immune cell infiltrates in iron-induced murine ccRCC.	**18-24**	**0%**
Subtask 1: T cell and macrophage immune cell infiltrate subtyping by flow cytometry	18	0%
Subtask 2: Mouse tissue IHC and scoring for T cell and macrophage markers	19-22	0%
Subtask 3: Statistical analyses for subtasks 1 and 2	23-24	0%
Milestone Achieved: **Comparison of tumor immune cell infiltrate density and subtypes among *vhl*$^{+/+}$, *vhl*$^{+/-}$ or *vhl*$^{-/-}$ mouse cohorts**	**24**	**0%**
Specific Aim 3: Determine the role of HIF-2α in VHL inactivation-mediated iron dependency of human ccRCC cells.		
Aim 3, Major Task 1: Determination of HIF-2α requirement for iron dependency in human VHL-mutated ccRCC cell lines.	**1-18**	**90%**
Subtask 1: Generation of clear cell renal cell carcinoma (ccRCC) cell lines with ectopic HIF-2α overexpression Cell lines: 786-0 [ATCC], RCC4 [ECACC]	1-5	100%
Subtask 2: Measure and analyze effects of HIF-2α overexpression on ccRCC apoptosis and cell cycle arrest in response to iron chelator drug treatment Cell lines: 786-0 [ATCC], RCC4 [ECACC]	6-17	0%
Milestone Achieved: **Determination of effects of HIF-2α overexpression on ccRCC apoptosis and cell cycle arrest rates in response to iron deprivation**	**17**	**60%**
Subtask 3: Measure and analyze effects of HIF-2α knockdown on ccRCC cell apoptosis and cell cycle arrest in response to iron chelator drug treatment Cell lines: 786-0 [ATCC], RCC4 [ECACC]	1-18	100%
Milestone Achieved: **Determination of effects of HIF-2α knockdown on ccRCC apoptosis and cell cycle arrest rates in response to iron deprivation**	**18**	**100%**
Aim 3, Major Task 2: Correlation of HIF-2α expression and iron levels in ccRCC patient tissues.	**1-24**	**100%**
Subtask 1: Procurement of first 10 of 20 nephrectomy patient tumor tissues	1-12	100%
Subtask 2: Procurement of last 10 of 20 nephrectomy patient tumor tissues	13-24	100%
Subtask 3: Measurement and statistical correlation of HIF-2α and free iron levels in dissociated cancer cells of tumors prospectively procured from ccRCC patients	1-24	100%

Milestone Achieved: **Correlation between free iron levels and HIF-2α protein levels in ccRCC patient tumor tissues**	**24**	**100%**
Subtask 4: IHC of HIF-2α protein in ccRCC patient tissue microarray	1-4	25%
Subtask 5: Statistical correlation between HIF-2α and total iron levels in the ccRCC TMA	5-6	0%
Milestone Achieved: **Correlation between total iron levels and HIF-2α protein levels in ccRCC patient tumor tissues**	**6**	**25%**

What was accomplished under these goals?

Specific Aim 1

Major Activities: The major activity accomplished for Specific Aim 1 was mouse breeding and generation of genetically engineered mice harboring both the Pax8-Cre promoter and a floxed Vhl allele within the same mouse, to yield a kidney-specific Vhl allele knockout. We also began and completed the 3-month daily intraperitoneal FeNTA (iron) injection treatments in these genetically engineered mice, and in control mice harboring wild-type Vhl in kidneys. Specific technical challenges and associated delays were encountered, as detailed below. Accordingly, we were not able to generate and treat as many of these mice as proposed, and breeding/treatments are still ongoing.

Specific Objectives: The specific objectives of this Aim were 2-fold: 1) to generate genetically engineered mice lacking *Vhl* in the mouse kidneys; and 2) to perform iron treatments to induce kidney tumors in these genetically engineered mice via iron ("FeNTA") treatment in order learn how *Vhl* loss alters the incidence, time-frame and histopathology of renal tumorigenesis.

Significant Results: Mice with Pax8-Cre underwent multiple rounds of breeding with floxed Vhl mice, as per the ACURO-approved and IACUC-approved protocol. In the prior reporting interval, breeding was delayed by multiple challenges. For the current reporting period, breeding was further delayed by the unexpected new discovery of **low fertility among male mice** when simultaneously harboring both the Pax8-Cre and floxed Vhl alleles. Less than 20% of these males successfully impregnated female mice during harem breeding with female mice. Furthermore, <10% of these male mice gave rise to >1 pregnancy. Although the cause of male infertility remains unclear, necropsy demonstrated that these mice have bilaterally abnormal epididymes suggestive of epididymal tumors (**Figure 1**). Epididymal tumors are well known to occur in humans who inherit a single mutant VHL allele, and these human epididymal tumors can cause infertility when bilateral. However, there is no previously described animal model of VHL mutation-induced epididymal tumors. Accordingly, our discovery may have translational significance by providing a novel animal

model of VHL-mutation induced male infertility secondary to bilateral epididymal tumors, however future research will be necessary to explore the histopathology of these epididymal lesions to confirm neoplastic change. In the interim, the unexpected challenge of infertility among male mice with the Pax8-Cre/floxed-VHL genetic alterations had two significant consequences: (1) additional delay in breeding; and (2) generation of a total of only 35 male mice with kidney-specific homozygous Vhl knockout for FeNTA (iron) treatment, rather than the targeted number of 50 male mice for this cohort.

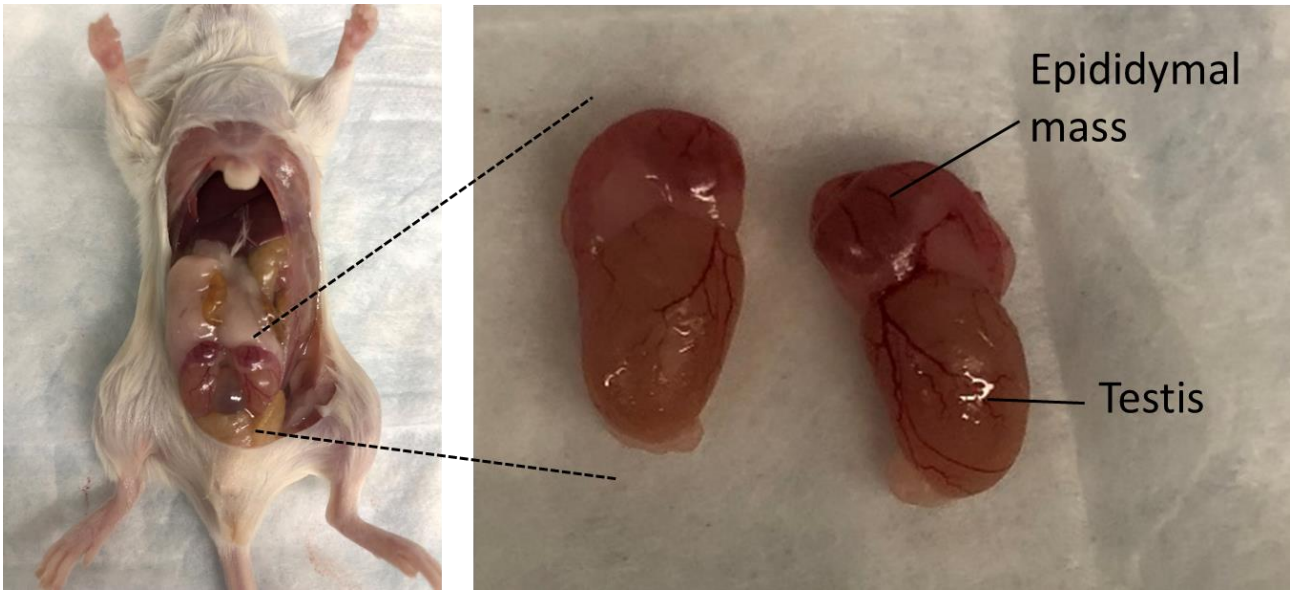


Figure 1. Representative bilateral epididymal masses in a Pax8-Cre+/floxed-Vhl male male mouse.

A second major challenge encountered for Specific Aim 1 during the reporting period was that FeNTA iron treatments were poorly tolerated among mice with kidney-specific homozygous Vhl knockout. Of 35 mice with kidney-specific homozygous Vhl knockout that underwent FeNTA treatment to induce renal iron overload, most required dose reductions or holding during the treatment period, and only 17 (49%) survived treatment. Among these 17 surviving mice, only 3 completed treatment without dose holding. In comparison, the vast majority of control mice (without homozygous Vhl knockout) survived treatment without drug dose holding or reductions, as expected. This observation of poor treatment tolerance in mice with Vhl loss is significant because it supports that Vhl gene expression in the mouse kidney introduces heightened sensitivity to renal iron overload. All mice finished iron treatment between June 2022 and Sept 2022. Whether the interplay between renal Vhl expression and renal iron overload will translate into a higher rate of renal tumorigenesis should become evident over the next 6-9 months (March 2023-June 2023). 6-month post-treatment MRI scans will be performed in December 2022 through March 2023.

Specific Aim 2

No work was performed for the reporting period for Specific Aim 2, which is scheduled to be performed in Winter-Spring 2023, since this work requires a 9 month delay from the completion of iron treatments.

Specific Aim 3

Major Activities. Major activities for Specific Aim 3 accomplished during the reporting period include:

1. Successful lentiviral infection of the 786-0 and RCC4 human ccRCC cell lines with a HIF-2α overexpression plasmid lacking an iron response element (IRE) to generate ccRCC cell lines with HIF-2α activity that is dissociated from its usual dependence on iron.
2. Procurement of 12 additional ccRCC patient tumors and generation of single cell suspensions to achieve the target total of 20 procured tumors over 2 years.
3. Evaluation of free iron levels relative to HIF-2α protein expression levels using imaging flow cytometry in single cell suspensions from 20 ccRCC patient tumors.
4. Obtainment of new HIF-2α antibody developed by an outside lab with internationally renowned expertise in HIF-2α immunohistochemistry.

Specific Objectives and Significant Results:

1. *Specific Objective #1*: We have previously shown that iron depletion induces apoptosis and cell cycle arrest in ccRCC cell lines (786-0, RCC4) but not in benign renal cells, indicating that ccRCC have a unique dependency on iron for cell cycle progression and apoptosis escape, however the mechanisms underlying this dependency are unknown. Our hypothesis is that ccRCC dependency on iron arises due to the required expression of HIF-2α in ccRCC cells (but not benign renal cells) and the reliance of this HIF-2α expression on iron. This hypothesis predicts that forced overexpression of HIF-2α without its usual iron response element (IRE) (normally found within the 5' HIF-2α transcript) will prevent iron depletion from inducing apoptosis and cell cycle arrest in ccRCC cells, since HIF-2α expression will no longer depend on iron. Therefore, our specific objective was to generate ccRCC cell lines with ectopic overexpression of HIF-2α transcript lacking an IRE (Major Task 1, Subtask 1), and to determine whether this alteration blocks apoptosis and cell cycle arrest from occurring in response to iron depletion.

   Significant Results: In the prior reporting period, we unsuccessfully attempted to stably transfect two ccRCC cell lines (786-0 and RCC4) with a HIF-2α-overexpression plasmid lacking an IRE, or with an empty vector (EV) plasmid as control; we also unsuccessfully attempted transient transfection, including under several different conditions and using different HIF-2α-overexpression plasmids. In the current reporting period, we continued to trouble shoot through these approaches without initial success. We then modified our overexpression approach by using instead a lentiviral infection strategy (**Figure 2**). As seen in Figure 2, this strategy was successful in generating cell lines with stable ectopic overexpression of HIF-2α.

Our next step will be to treat these cell lines iron chelator drug to induce iron depletion, and to assess the effect of this iron depletion of rates of apoptosis and cell cycle arrest.

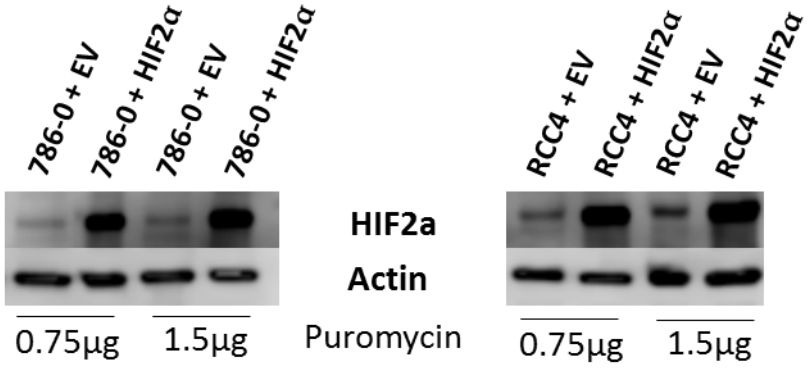


Figure 2. Generation of ccRCC cell lines with HIF-2α overexpression. Representative Western Blots of 786-0 RCC4 ccRCC cell lines after infection with commercial HIF-2α overexpression vector (EX-M0910-Lv105) lacking the normal HIF-2α iron response element (IRE) sequence. Infected 786-0 and RCC4 cell lines were selected in puromycin antibiotic-supplemented media at 2 different concentrations (0.75ug and 1.5ug).

2. *Specific Objective #2*: Our hypothesis that ccRCC cell dependency on iron is mediated by HIF-2α dependency on high iron levels predicts that HIF-2α protein levels and free (oxidized) iron levels will correlate positively with each other. We therefore attempted to determine whether HIF-2α protein levels and free iron levels are positively correlated within different cancer cells of any individual patient, and among tumors from different patients.

   Significant Results: In the previous reporting period, we procured kidney tumors from 8 ccRCC patient tumors, from which single cell suspensions were generated for evaluation of free iron levels and HIF-2α protein levels. In the current reporting period, we procured an additional 12 ccRCC patient tumors and similarly harvested single cell suspensions, reaching the target total of 20 ccRCC patient tumor single cell preps. Single cell preps from all 20 tumors were analyzed by multimarker imaging flow cytometry to determine the relation between free iron levels and HIF-2α protein levels. Prior to imaging flow cytometry, tumor cell preps were stained with Phen Green, the fluorescence of which is inversely proportional to free iron level, in addition to the DRAQ5 nucleus marker and non-overlapping fluorophore-tagged antibodies to HIF-2α, vimentin (ccRCC cell marker), CD31 (endothelial cell marker) and CD45 (pan-immune cell marker) (**Figure 3**). Phen Green is a fluorescently tagged metal chelator whose staining level correlates inversely with free iron levels, whereas vimentin allows identification of cancer cells, while CD45 and CD31 are cell membrane markers that allow for exclusion of tumor-infiltrating immune cells and endothelial/vascular cells, respectively.

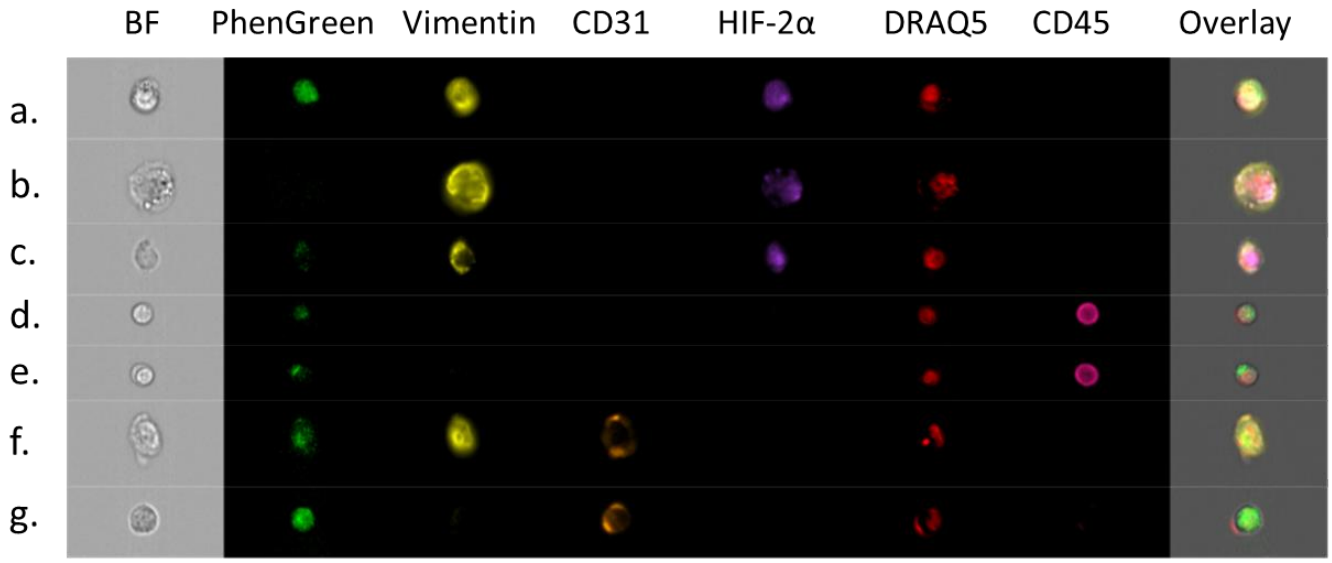
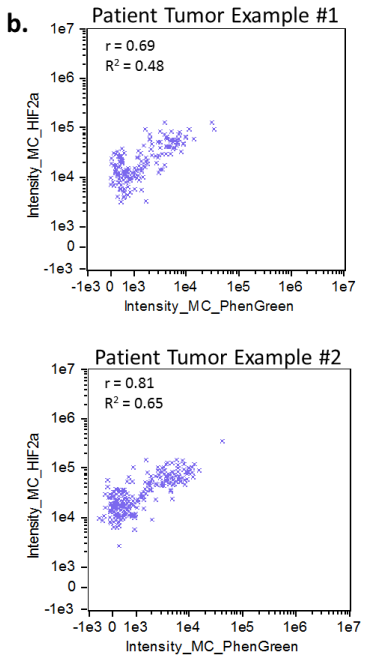


Figure 3. Measurement of free iron levels and HIF-2α protein levels in cancer cells of ccRCC patient tumors. Imaging flow cytometry was performed on single-cell suspensions prepared from n=20 ccRCC patient tumors after staining with a multi-marker cocktail consisting of the fluorescent stains, Phen Green and DRAQ5, in addition to fluorophore-tagged antibodies against HIF-2α, vimentin, CD31 and CD45. Tumor-infiltrating immune cells were identified by CD31+ or CD45+ stain and excluded, while cancer cells were identified by a vimentin+/CD31-/CD45- staining pattern. Nuclear HIF-2α stain in cancer cells was visually confirmed by overlap with the nuclear marker stain DRAQ5. Phen Green levels are inversely proportional to iron levels (high Phen Green levels indicate low iron levels, and vice versa). **(a-c):** Representative imaging flow cytometry images of an individual cancer cell with high (a), absent (b), or low (c) Phen Green levels. (**d, e**): Representative imaging flow cytometry images of an immune cell. **(f, g):** Representative imaging flow cytometry images of an endothelial cell with (f) or without (g) vimentin expression. Each row represents an individual cell.

For each of the 20 tumors, cancer cells identified by a vimentin+/CD31-/CD45- staining pattern demonstrated a positive correlation between HIF-2α nuclear protein levels and Phen Green levels (**Figure 4a, b**). The positive correlation ranged in strength from weak to very strong, with most (15/20, 75%) cases having at least a moderate strength of correlation (**Figure 4a**). Furthermore, hen the mean fluorescent intensity of staining for HIF-2α and Phen Green for each tumor was compared among all 20 tumors, we detected a non-significant trend (p=0.059) toward a strong positive correlation between mean HIF-2α levels and mean Phen Green levels (**Figure 4c**). Because Phen Green levels are inverse to free iron levels, we conclude that HIF-2α and iron levels are not positively correlated in ccRCC cells as predicted by the hypothesis, and may be negatively correlated. This finding suggests that high HIF-2α levels are not dependent on high free iron levels, and argues against the hypothesis that ccRCC dependency on iron is mediated by HIF-2α dependency on iron.

a.

Sample #	Correlation of Phen Green vs. HIF-2α levels		
	R value	Positive or negative	Strength
1	0.44	positive	moderate
2	0.60	positive	strong
3	0.31	positive	moderate
4	0.80	positive	very strong
5	0.21	positive	weak
6	0.85	positive	very strong
7	0.42	positive	moderate
8	0.42	positive	moderate
9	0.64	positive	strong
10	0.65	positive	strong
11	0.36	positive	weak
12	0.34	positive	weak
13	0.28	positive	weak
14	0.64	positive	strong
15	0.69	positive	strong
16	0.77	positive	strong
17	0.20	positive	weak
18	0.50	positive	moderate
19	0.81	positive	very strong
20	0.62	positive	strong



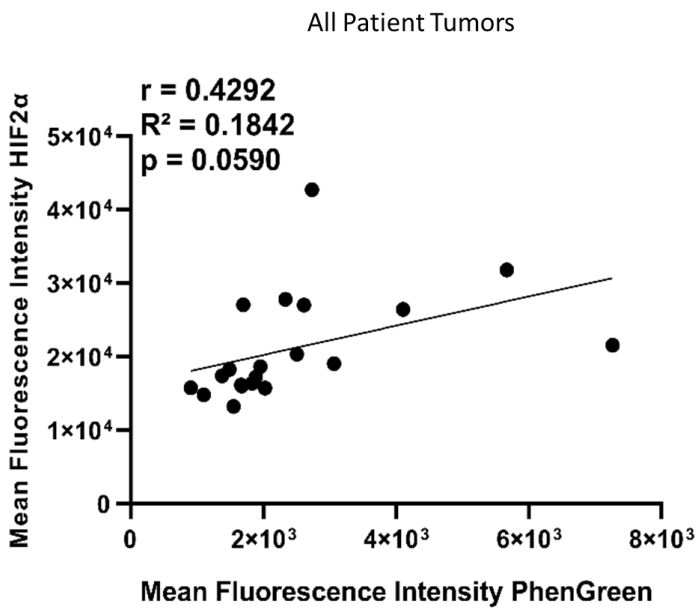


Figure 4. Positive correlation of Phen Green levels and HIF-2α levels in cancer cells isolated from ccRCC patient tumors. Imaging flow cytometry was performed on single-cell suspensions prepared from n=20 ccRCC patient tumors after staining with a multi-marker cocktail consisting of the fluorescent stains, Phen Green and DRAQ5, in addition to fluorophore-tagged antibodies against HIF-2α, vimentin, CD31 and CD45. Phen Green levels are inversely proportional to iron levels (high Phen Green levels indicate low iron levels, and vice versa). **(a)** The R-value and corresponding strength of correlation is showing for each tumor. (**b**) Relation between HIF-2α and Phen Green levels in cancer cells from 2 representative patients; each plotted dot represents an individual cancer cell. **(c)** Relation between mean HIF-2α levels and mean Phen Green levels across different patient tumors (n=20); each plotted dot represents an individual tumor.

3. *Specific Objective #3*: The hypothesis (that ccRCC cell dependency on iron is mediated by HIF-2α dependency on high iron levels) predicts that HIF-2α levels will correlate not only with free (reactive/oxidized) iron levels, but also with total iron levels in ccRCC cells. Whereas Specific Objective #2 evaluated the relation of HIF-2α and free iron levels, Specific Objective #3 was to evaluate the relation of HIF-2α and total iron levels. The goal was to determine whether HIF-2α protein levels and total iron levels are positively correlated among tumors from different patients. The plan was to do this by performing immunohistochemistry (IHC) to measure HIF-2α levels in a RCC patient tissue microarray that has been already stained for total iron levels.

   Significant Results: In the prior reporting period, we encountered several technical challenges in performing HIF-2α IHC using multiple commercial HIF-2α antibodies under a variety of different conditions. We discussed this challenge with world expert in HIF-2α biology, Dr. Volker Haase (Vanderbilt University), who shared in personal communication that commercially available HIF-2α antibodies uniformly fail to work reliably. Based on Dr. Haase's recommendation, we reached out to a U.K. investigator who is similarly a renowned researcher of HIF-2α (Dr. Peter Ratcliffe, Oxford University), who previously described HIF-2α IHC staining in ccRCC patient tissues using a reliable HIF-2α antibody that is not commercially available. During the current reporting period, we have worked with Dr. Ratcliffe's lab to complete the necessary Material Transfer Agreement and receive this antibody, which we now have in hand. However, before our receipt of the antibody, the commercial antigen retrieval kit used by Dr.

Ratcliffe's lab for this antibody was discontinued, and is no longer available; and the aliquot of antibody that we received from Dr. Ratcliffe's lab is inadequate to test new antigen retrieval kits and perform optimization. Accordingly, Dr. Ratcliffe's lab is currently working with new antigen retrieval kits, with plans to inform us when they have optimized a new antigen retrieval kit, which we will then use. We expect this to be performed and completed within this next year, so that we may move forward with HIF-2α immunostaining to complete this Specific Objective.

What opportunities for training and professional development has the project provided?

1. ***Mentee Opportunities*.** A highlight of project has been my partnership with my primary mentor, Dr. Gross, who has abundant mouse modeling experience as former director of the transgenic animal modeling core at our institute. This past 2 years have afforded invaluable training experience in the form of monthly didactic one-on-one sessions with Dr. Gross. I have complemented these sessions with quarterly meetings with co-mentor, Dr. Mohler, who has advised me on how to balance my laboratory work with my clinical duties as a surgeon scientist.

2. ***Manuscript Preparation and Publication***. This award year afforded me protected research time to compose a manuscript on iron levels in kidney cancer patient tissues, which was published in *Frontiers in Oncology*.

3. ***Training in genomic bioinformatics***. My biomarker research interests benefited from dedicated training in genomics bioinformatics via monthly didactic sessions between with collaborator and bioinformatician, Dr. Eng. Dr. Eng has instructed me on the basic of bioinformatics applications in different aspects of kidney cancer research.

4. ***Attendance and participation at academic conferences*.** This research grant has afforded me opportunity to attend 5 national conferences in Urology, including serving as a moderator at the International Kidney Cancer Symposium (annual meeting of the Kidney Cancer Association), and multiple podium presentations at the annual conferences of the American Urological Association (AUA). I also attended the annual meetings of the Northeast Section of the American Urological Association. These conferences each afforded me excellent opportunity for professional networking with other kidney cancer investigators.

5. ***Seminar attendance***. This DoD research award also afforded me protected academic time to regularly attend several seminar series at my institute, including Roswell Park Medical Grand

Rounds, and the Cancer Genetics and Genomics Seminar Series. Through these seminars, I was able to see Visiting Professor Lectures from world-renowned cancer researchers, such as most recently City of Hope kidney cancer clinician scientist, Sumatra Pal, MD.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Specific Aim 1:

Task 1: **Determine *vhl* inactivation effect on iron-induced mouse tumor burden using MRI**

- Subtask 1: We will complete mouse breeding by November 2022. My goal is to increase the surviving number of Pax8-Cre/floxed-Vhl mice from 17 to approximately 25.
- Subtask 2: For any new Pax8-Cre/floxed-Vhl mice that we are able to generate in the next 3 months, we will perform a second/round of FeNTA (iron) mouse treatments, which will be completed by March 2023.
- Subtask 3-6: MRI evaluation of kidneys from mice who have completed FeNTA (iron) treatment will be performed and analyzed by Winter 2023, and MRI's for the second round of FeNTA-treated mice will be completed and analyzed by Fall 2023.

Task 2: **Assess HIF-α dysregulation and iron accumulation in iron-induced mouse kidney tumors**

- Subtask 1: We will harvest mouse kidney tumors from the first round of mice completing FeNTA treatment in Spring 2023
- Subtask 2-3: We will complete the assay in these mouse tumors for HIF-α pathway protein levels and iron levels by Summer 2023

Task 3: **Histopathologic comparison among mouse cohorts.**

- Subtasks 1-3: We will complete the histopathologic determination of tumor incidence, number, size and histology, including comparisons between mouse cohorts, by Fall 2023

Specific Aim 2:

Task 1: **Determine effect of *vhl* inactivation on the genomic profile of iron-induced murine ccRCC**

- Subtasks 1-2: We will perform whole exome sequencing and RNAseq on mouse tumor tissues by Spring 2023
- Subtask 3: We will complete the sequencing analyses including comparisons between cohorts by Summer 2023

Task 2: **Determine effect of *vhl* loss on density and subtypes of immune cell infiltrates in iron-induced murine ccRCC**

- Subtasks 1-3: We will complete the evaluation of immune cell infiltrate in tumors using flow cytometry and immunohistochemistry by Summer 2023

Specific Aim 3:

Task 1: **Determine effect of *vhl* inactivation on the genomic profile of iron-induced murine ccRCC**

- Subtask 2: We will perform apoptosis assays and cell cycle assays in our genetically engineered ccRCC cells harboring overexpression of a HIF-2α transcript lacking an IRE (iron response element), which will be completed by Winter 2023.

Task 2:

- Subtask 3: We will use flow cytometry to measure the correlation between HIF-2α and free iron levels in procured tumor specimens by Summer 2022.
- Subtasks 4-5: We will obtain the new non-commercial HIF-2α antibody for immunohistochemistry, and perform immunohistochemistry for HIF-2α during Fall 2022, with analyses completed by Winter 2023.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Nothing to Report for changes in approach

Actual or anticipated problems or delays and actions or plans to resolve them

1. ***Delay in initiation of mouse breeding and iron treatments*.** There were several challenges encountered during the prior reporting period and current reporting period, that each contributed to a delay in completion of mouse breeding, which in turn resulted in a delay of mouse iron treatments. The delays specific to the current reporting period included:

    i. PAX8-Cre mice were obtained once the floxed Vhl mice were ready, but there were initial problems confirming the genotype identity of these mice. Technical challenges with PAX8-Cre genotyping later resurfaced during breeding. What was suspected initially to be low transmission rate to offspring of the PAX-Cre alteration was subsequently determined to be due to technical inconsistency with the genotyping PCR reaction, as described in detail in Section 3 ("Accomplishments") above. Ultimately, genotyping was switched from being performed by our Genotyping shared resource core facility to being outsourced to a 3rd party commercial genotyping company (Transnetyx, Inc.) that has since partnered with our mouse core facility to provide genotyping resources to the entire institute. We have found this new resource to be consistent and reliable.

    ii. We observed a low rate of pregnancies among male breeding mice who were positive for both PAX8-Cre and floxed VHL alleles. The cause of this unanticipated finding is unclear and not previously reported in studies using PAX8-Cre to drive excision of the VHL gene using a Cre-Lox recombination approach. We have harvested genital organs from these male mice for potential future research to investigate the cause of low male fertility.

    Plans for resolution. The delays in mouse breeding/treatment resulted in delay to initiate mouse iron treatments in Specific Aim 1 and delay to harvest mouse tumor assays in Specific Aim 2. We expect these delays to result in an **overall 1-year delay**, for which we have requested and been granted a one-year no-cost extension to complete the proposed work.

2. ***Poor mouse tolerance of iron treatment.*** There was an unexpected poor tolerance of iron treatment in mice with VHL gene loss targeted to the kidney (but not in other mouse groups/controls). Most mice with kidney-specific homozygous VHL loss did not survive treatment, compared to a minority of mice in other groups. This poor tolerance, combined with

the delays in breeding, resulted in only 17 mice with kidney-specific VHL loss that have completed iron treatments, which is one third of the proposed cohort size for this arm (n=50).

Plans for resolution. We expect to generate an additional ~8 with homozygous VHL loss over the next 3 months to bring the total mouse number for this cohort up to 25. If these mice have higher propensity for renal tumorigenesis (as predicted by our hypothesis), such that they generate renal tumors at a frequency of at least 40% (rather than the usual 20%), then a total number of 25 mice in this cohort arm (rather than 50) may be sufficient to test the hypotheses of Specific Aim 1 and to yield at least 10 mouse renal tumors (needed to complete Specific Aim 2). Hence, the lower number of total mice in this arm may turn out to be inconsequential.

3. ***Delay in HIF-2α immunohistochemical staining of ccRCC patient tumors*.** Technical challenges resulting in this delay are described in detail in Section 3 (Accomplishments). This delay is not expected to have a significant impact.

   Plan for resolution: A non-commercial antibody with strong published track record of reliable immunohistochemical staining in formalin-fixed paraffin embedded tumor sections has been acquired from an outside laboratory (Dr. Peter Ratcliffe, Oxford). This outside lab is currently working with new antigen retrieval kitsf or this antibody, with plans to inform us when they have optimized a new antigen retrieval kit, which we will then use. We expect this to be performed and completed within this next year.

4. **Delay in overexpression of IRE-lacking HIF-2α protein in ccRCC cell lines.** The technical challenges resulting in this delay are described in detail in Section 3 (Accomplishments). This delay is not expected to have a significant impact.

   Plan for resolution: The challenge of establishing these genetically engineered cell lines has been overcome, as described in Section 3. Measurement of the effects of iron depletion on apoptosis and cell cycle arrest in these cell lines will be completed by Winter 2023.

Changes that had a significant impact on expenditures

No changes have had any impact on planned expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Christopher J Greene, Kristopher Attwood, Nitika J Sharma, Benjamin Balderman, Rongia Deng, Jason B Muhitch, Gary J Smith, Kenneth W Gross, Bo Xu, Eric C Kauffman. Iron accumulation typifies renal cell carcinoma tumorigenesis but abates with pathological progression, sarcomatoid dedifferentiation, and metastasis. *Front Oncol*. 2022 Aug 5;12:923043. doi: 10.3389/fonc.2022.923043

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Eric Kauffman
Project Role:	Principle Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	Dr. Kauffman supervises the overall project and is responsible for fulfilling the project goals.
Funding Support:	

Name:	Nitika Sharma
Project Role:	Technologist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	18
Contribution to Project:	Ms. Sharma was responsible for the technical performance and analysis of all mouse breeding, iron treatments and patient tissue/cell assays described in this progress report under Dr. Kauffman's supervision.
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWAR

Not applicable

QUAD CHARTS:

Not applicable

9. APPENDICES:

Nothing to report.