Activation of HIF2α in Kidney Proximal Tubule Cells Causes Abnormal Glycogen Deposition but not Tumorigenesis

Leiping Fu1,4, Gang Wang1,4, Maria M. Shevchuk2,4, David M. Nanus3,4, and Lorraine J. Gudas1,4

Abstract

Renal cell carcinoma (RCC) is the most common primary cancer arising from the kidney in adults, with clear cell renal cell carcinoma (ccRCC) representing approximately 75% of all RCCs. Increased expression of the hypoxia-inducible factors-1α (HIF1α) and HIF2α has been suggested as a pivotal step in ccRCC carcinogenesis, but this has not been thoroughly tested. Here, we report that expression of a constitutively activated form of HIF2α (P405A, P530A, and N851A, named as HIF2αM3) in the proximal tubules of mice is not sufficient to promote ccRCC by itself, nor does it enhance HIF1αM3 oncogenesis when coexpressed with constitutively active HIF1αM3. Neoplastic transformation in kidneys was not detected at up to 33 months of age, nor was increased expression of Ki67 (MKI67), γH2AX (H2AFX), or CD70 observed. Furthermore, the genome-wide transcriptome of the transgenic kidneys does not resemble human ccRCC. We conclude that a constitutively active HIF2α is not sufficient to cause neoplastic transformation of proximal tubules, arguing against the idea that HIF2α activation is critical for ccRCC tumorigenesis. Cancer Res; 73(9); 2916–25. ©2013 AACR.

Introduction

Renal cell carcinoma (RCC) is the most common primary cancer arising from the kidney in adults, with clear cell renal cell carcinoma (ccRCC) representing approximately 75% of all RCCs (1, 2). Loss of expression or mutation of the von Hippel-Lindau (VHL) tumor suppressor gene is found in hereditary and most sporadic ccRCCs (1, 3). This suggests an etiologic role for VHL gene loss in renal carcinogenesis. However, the exact pathway by which loss of VHL leads to ccRCC has not been elucidated. The best studied and likely the most important effect of VHL loss is the increased expression of the hypoxia-inducible factor-1α (HIF1α) and hypoxia-inducible factor-2α (HIF2α) (ref. 4–6). Increased expression of these 2 transcription factors has been proposed as a key step in ccRCC carcinogenesis (4). HIF2α shares approximately 48% amino acid homology with HIF1α (7). Both HIF1α and HIF2α are regulated by prolyl hydroxylation at proline sites and by asparaginyl hydroxylation at an asparagine site under normoxic conditions (7), and each pairs with HIF1β and binds to hypoxia responsive elements (HREs, 5′-RCGTTG-3′, ref. 7). HIF2α protein levels are increased and HIF2α is transcriptionally activated in VHL−/− renal carcinomas (8). Furthermore, elimination of HIF2α in RCC cell lines is sufficient to suppress VHL−/− tumor growth in xenograft models (9, 10). Tumor suppression by pVHL can be overridden by HIF2α but not HIF1α in tumor xenografts of cultured 786-0 cells (9, 11, 12). These data collectively suggest that HIF2α may be more oncogenic than HIF1α for tumor xenograft growth, although other data indicate that HIF1α is more oncogenic (13–18).

At present, few murine models that exhibit the pertinent features of human ccRCC exist. Human tumorgraft models of ccRCC have significant limitations, including the immunodeficient state of the animals (19). We recently reported a transgenic model of cancer of the kidney (TRACK) model, which mimics early-stage human ccRCC through expression of a triple mutant (P402A, P564A, and N803A) human HIF1α construct (13). We have now used the same kidney proximal tubule specific type 1 γ-glutamyl transpeptidase (GGT or γGT) promoter (20, 21) to drive expression of a triple mutant (P405A, P530A, and N851A), constitutively active HIF2α protein specifically in the proximal tubule cells (PTC) to determine if this results in carcinogenesis. We show here that this triple mutant, HIF2α protein (HIF2αM3) is active, even under normoxic conditions. Transgenic mice that express this triple mutant, constitutively active HIF2α construct specifically in the kidney exhibit glycogen accumulation and hydropic degeneration, but no lipid deposition. We also do not observe renal cysts or disorganized proximal tubules resembling in situ carcinoma. We do not observe overexpression of molecular markers of cancer, for example, Ki67, γH2AX, and CD70, in the kidneys of HIF2αM3 transgenic positive (TG+) mice. Furthermore, we analyzed entire transcriptomes of cells from the HIF2αM3 TG+ kidney cortex by Next Generation Sequencing/RNA-seq. The kidney cortex transcriptome of HIF2αM3 TG+ mice does not...
HIF2α Does Not Cause Kidney Tumorigenesis

not closely resemble that of human ccRCC, consistent with the lack of tumorigenesis in these mice.

Materials and Methods

**Plasmid construction and generation of transgenic mice**

Mutated, constitutively active mouse HIF2α cDNA was created by site-directed mutagenesis (Invitrogen) of conserved proline residues (proline 405 and 530) and a conserved asparagine (asparagine 851) into alanine residues. The rat GGT promoter (−1930 to +246) was amplified by PCR from a plasmid (21). The GGT promoter, mutated HIF2α, and β-globin poly-A were cloned into pBlueScript and named γGT-HIF2α triple mutant (γ-HIF2αM3).

A linearized XhoI-XbaI fragment (vector sequence removed) was injected into pronuclei of one-cell embryos (C57BL/6 x C57BL/6) at the Weill Cornell Medical College (WCMC) Mouse Genetics Core. Southern Blot analysis was then conducted (13). The γ-HIF2αM3 transgene was carried in the heterozygous state. The γ-HIF2αM3-1 and the γ-HIF2αM3-17 lines were mated with the TRACK mice to obtain γ-HIF2αM3; γ-HIF2αM3 double TG+ mice. Both γ-HIF2αM3 and γ-HIF2αM3 transgenes are carried in the heterozygous state in the double TG+ mice. All animal procedures were conducted following guidelines of the Weill Cornell Research Animal Resource Center.

**Tissue dissection, processing, pathological review, and histological staining**

Tissues were fixed, processed, sectioned, and hematoxylin and eosin stained (13). Slides were reviewed in a blinded manner by Dr. Shevchuk, an experienced clinical pathologist specializing in human kidney cancer, and independently by a veterinary pathologist, Dr. Linda Johnson, from the Laboratory of Comparative Pathology, WCMC. Immunohistochemistry was carried out as described (13). Antibodies used: HIF2α (100–122, Novus Biologicals); CA-IX (sc-25600, Santa Cruz); Glut-1 (ab14683, Abcam); Ki67 (M7249, Dako); and γH2AX (9718S, Cell Signaling). Periodic acid-Schiff (PAS) stain was conducted on paraffin-embedded and cryopreserved sections (13). Oil red O (ORO) staining was performed as described (13).

**Reverse transcriptase PCR, whole genome RNA sequencing, and data analysis**

Total RNA was extracted using miniRNAeasy columns (Qiagen). Semiquantitative reverse transcriptase (RT)-PCR was then carried out (13). Total RNA from thin, outer slices of kidney cortex was used for whole genome sequencing. The complete transcriptomes of kidney cortex from 3 γ-HIF2αM3 18-month-old TG+ male mice and 3 age-matched wild-type (WT) C57BL/6 male mice were sequenced on an Illumina HiSeq2000 Sequencer. The reads were aligned to the mouse genome (NCBI37.55/MM9) using the Burrows-Wheeler Aligner (BWA; ref. 22) in GobyWeb software (23). Comparisons of gene expression changes between γ-HIF2αM3 TG+ and WT male mice were carried out using differential expression analysis with Goby in the GobyWeb. Benjamini and Hochberg FDR adjustment (q value) for Student t test (Student t test-BH-FDR-q value) and Benjamini and Hochberg FDR adjustment (q value) for Fisher exact test (Fisher exact test-BH-FDR-q value) were used to determine statistical significance. The data have been in the GEO database (accession no. GSE45254).

**Statistical analysis**

Results are expressed as the mean ± SEM. Student t test was used to determine the statistical significance of the γH2AX+ and Ki67+ cell number differences between TG+ and WT kidneys.

**Results**

**Generation of transgenic mice that express mutated, constitutively active HIF2α**

To examine the role of HIF2α in ccRCC carcinogenesis, we constructed a GGT-HIF2α triple-mutant plasmid (γ-HIF2αM3, Fig. 1A). After confirmation of activity in a cultured normal kidney proximal tubule cell line, a linearized γ-HIF2αM3 XbaI-XhoI fragment was used to generate γ-HIF2αM3 transgenic mice. A total of 10 of 50 founder mice harbored the integrated target gene by Southern analysis (Fig. 1B, founders #17 to #21). Three lines (#21, #34, and #38) did not show germ line transmission. The other 7 lines were evaluated by RT-PCR, using a transgene-specific primer pair (primers 1 and 2, Fig. 1A) for transgene mRNA levels in the kidney, spleen, liver, heart, lung, intestine, skeletal muscle, and testis/ovary. The triple-mutant HIF2α (γ-HIF2αM3) was expressed only in the kidneys of TG+ lines #1, #17, #27, #31, and #48 (Fig. 1C, TG+, #1 as an example). The transgene was not expressed in the other organs analyzed, except for low expression in the testis (Fig. 1D). VHL, endogenous HIF1α, and endogenous HIF2α mRNA levels were not changed in the kidneys of TG+ mice compared with the kidneys of transgenic negative (TG−) mice (Fig. 1C, TG−). All 5 TG+ founder lines, γ-HIF2αM3-1, γ-HIF2αM3-17, γ-HIF2αM3-27, γ-HIF2αM3-31, and γ-HIF2αM3-48, developed normally and could pass the transgene to offspring following a Mendelian pattern of inheritance. Because the GGT promoter is not active until about 3 weeks after birth (20, 21), we did not expect and did not observe any gross developmental abnormalities in the kidneys.

**HIF2α and its target genes are upregulated in TG+ kidneys**

As described above, the HIF2α transgene mRNA is expressed in TG+ kidneys (Fig. 1C). We next examined the expression by semiquantitative RT-PCR of CA-IX (NP_647466) and Glut-1 (NP_055350), which are HIF target genes, and CD70 (TNFSF7), which is a marker of human ccRCC (24–27) but not a known HIF2α target gene. We detected increased mRNA levels of CA-IX and Glut-1, but not CD70 (Fig. 2A).

By immunohistochemistry, we confirmed increased HIF2α, CA-IX, and Glut1 protein staining in the abnormal proximal tubules (Fig. 2B, C, D, TG+), but not in the morphologically normal proximal tubules of the same γ-HIF2αM3 mice. As expected, weak or no detectable HIF2α, CA-IX, or Glut1 signals are observed in the proximal tubules of TG− and WT mice (Fig. 2B, C, D, TG−).
**γ-HIF2αM3-1 and γ-HIF2αM3-17 TG+ mice exhibit mild vacuolation**

Clear vacuoles were detected circumferentially around some, but not all, proximal tubule cell nuclei in the γ-HIF2αM3 mice. The γ-HIF2αM3-1 and γ-HIF2αM3-17 lines exhibited the strongest phenotype (Fig. 3A); the other lines had similar phenotypes, but displayed fewer proximal tubules containing clear vacuoles (data not shown). We identified vacuoles with a pale, eosinophilic to clear feathery cytoplasm without displacement of the nucleus, consistent with glycogen accumulation and hydropic degeneration (Fig. 3A, arrows) in one-year-old γ-HIF2αM3-1 and γ-HIF2αM3-17 mice. Increased numbers of vacuoles in some proximal tubules were observed in 2-year-old γ-HIF2αM3-1 and γ-HIF2αM3-17 mice (Fig. 3B, arrows). The histologically abnormal proximal tubule cells in TG+ mice were large, simple cuboidal epithelial cells (Fig. 3A and B), and clear vacuoles (data not shown). We identified two phenotypes, but displayed fewer proximal tubules containing clear vacuoles in some proximal tubules were observed in 2-year-old γ-HIF2αM3-1 and γ-HIF2αM3-17 mice (Fig. 3B, arrows). The histologically abnormal proximal tubule cells in TG+ mice were large, simple cuboidal epithelial cells (Fig. 3A and B), and these cells were surrounded by a tubular basement membrane, suggesting that these cells are under proper growth control. In ccRCC, the clear cytoplasm is caused by deposition of glycogen, phospholipids, and neutral lipids, particularly cholesterol esters (28). The cytoplasm of the abnormal cells in the HIF2α TG+ mice contained increased amounts of glycogen, as shown by PAS staining (Fig. 3C, arrows), but no increase in lipid as determined by ORO staining (Fig. 3D).

We examined 21 mice of the γ-HIF2αM3-1 line and 16 γ-HIF2αM3-17 TG+ mice and compared them with WT mice. The oldest examined was a 33-month-old TG+ male mouse. Six TG+ mice older than 24 months, 13 between the ages of 18 and 24 months, 9 between the ages of 12 and 18 months, and 9 younger than 12 months were analyzed. No renal cysts or disorganized proximal tubules resembling in situ carcinoma were identified in any of the 37 TG+ animals examined.

**Expression of markers of proliferation and DNA double strand breaks in the γ-HIF2αM3 TG+ mice**

Uncontrolled cell division/proliferation is one of the most prominent features of tumor cells. Genomic instability is another universal feature of tumor cells (29). An increased DNA mutation rate/genomic instability may cause neoplastic transformation (30). Ki67 is a marker for proliferation (31). The

![Figure 1. Generation of γ-HIF2αM3 transgenic mice. A, construction of the γ-HIF2αM3 plasmid. Only the fragment used to create the TG+ mice is shown. Three mutations (P405A, P530A, and N851A), dashed arrows. An intron (shaded square) was included in the β-globin poly A. Primers 1 and 2 were used to amplify the transgene by RT-PCR. B, Southern blot of the TG+ and TG− founders. Only founder #17 to #21 mice are shown. Founders #17 and #21 are TG+, others shown are TG−. C, HIF2α transgene, endogenous VHL, HIF1α, and HIF2α RT-PCR in kidneys of the γ-HIF2αM3-1 line. The HIF2α transgene was only detected in the TG+ mice. Endogenous VHL, HIF1α, and HIF2α mRNAs were expressed at similar levels in TG+ and TG− mice. β-Actin, loading control. D, HIF2α transgene RT-PCR in multiple organs of the γ-HIF2αM3-1 line mice. The HIF2α transgene was detected specifically in the kidneys and testes of TG+ mice from the γ-HIF2αM3-1 line. No expression of the γ-HIF2αM3 transgene was detected in any organ of the TG− mouse. β-Actin, loading control.]
serine 139 phosphorylated form of H2A histone family, member X (γH2AX) is a widely used marker that indicates double strand breaks (DSB). We examined Ki67 and γH2AX protein levels in TG+ versus TG− kidneys (Fig. 4). We detected few Ki67+ and γH2AX+ cells in WT proximal tubules (1.5 or 1.9 TG− cells/field, respectively). In the γ-HIF2αM3 TG+ mice, we observed statistically significantly decreased numbers of Ki67+ and γH2AX+ cells in the morphologically abnormal proximal tubules. There were 0.1 (P = 0.01, compared with TG−) and 0.3 (P = 0.02, compared with TG−) Ki67+ cells/field in the γ-HIF2αM3-1 and γ-HIF2αM3-17 TG+ kidneys, respectively; there were 0.9 (P = 0.03, compared with TG−) and 0.8 (P = 0.005, compared with TG−) γH2AX+ cells/field in the γ-HIF2αM3-1 and γ-HIF2αM3-17 TG+ kidneys, respectively. Thus, the kidneys from TG+ mice exhibited a reduction in Ki67 and γH2AX staining relative to WT.

The transcriptome of γ-HIF2αM3 TG+ kidneys does not closely resemble the transcriptome of human ccRCC

We next examined gene expression using next generation sequencing of the whole transcriptome (RNA-seq). We cut a thin slice of kidney cortex, which contains the preponderance of proximal tubules, and extracted total RNA for whole transcriptome analysis. High levels of proximal tubule marker mRNAs, for example, GGT, indicate kidney cortex identity.

We identified upregulation of 1,253 and downregulation of 749 transcripts in γ-HIF2αM3 TG+ versus WT kidney cortexes from 18-month-old mice using less stringent conditions (fold change > 1.2, Student t test-BH-FDR-q value < 0.05, Fisher exact test-BH-FDR-q value < 0.05). We identified upregulation of 206 and downregulation of 86 transcripts in γ-HIF2αM3 TG+ versus WT mice when data were filtered with a more stringent condition (fold change > 1.5, Student t test-BH-FDR-q value < 0.01, Fisher exact-test-BH-FDR- q value < 0.01). Certain HIF2α target genes, such as CA-IX, show significant overexpression in the HIF2αM3 TG+ versus WT samples. However, other putative HIF2α target genes, such as Oct4, cyclin D1, and TGFβ (32, 33), did not exhibit increased expression in the γ-HIF2αM3 TG+ versus WT kidneys. Cyclin D1 shows a fold change of 1.1 (Student t test-BH-FDR-q value = 0.49) and TGFβ transcripts show a fold change of 1.24, (Student t test-BH-FDR-q value = 0.19).

To compare the transcriptome of the γ-HIF2αM3 TG+ kidneys to human ccRCC, we identified the 20 genes most highly overexpressed at the RNA level in human ccRCC from Oncomine (Compendia Bioscience), combining 5 different datasets of human ccRCC patient samples (34–37). We identified a total of 5 datasets of cancer versus normal analysis of the ccRCC cancer type (Table 1). The 5 datasets we used are in Table 1. One of the 20 genes highly expressed in human ccRCC, EGLN3, shows significant upregulation in our γ-HIF2αM3 TG+ kidneys (> 3 fold; ref. Table 1). However, the remaining 19 genes that are highly overexpressed in human

![Image](https://example.com/image1.png)

**Figure 2.** Overexpression of HIF1α/HIF2α target genes in the γ-HIF2αM3 kidneys. Increased levels of HIF2α target genes, for example, CA-IX, Glut1, and H2AX, were observed in the abnormal proximal tubules of the TG+ mice. All scale bars represent 100 μm.

![Image](https://example.com/image2.png)

**Figure 3.** "Clear" cells in γ-HIF2αM3-43 mouse. Representative images of the histologic morphology (A and B), PAS stain (C), and ORO stain (D) of the "clear" cells observed in γ-HIF2αM3-1 and γ-HIF2αM3-17 male TG+ and TG− kidneys. Small vacuoles with a pale, eosinophilic to clear feathery cytoplasm without displacement of the nucleus were observed in 1-year-old TG+ mice as compared with WT mouse (B, arrow). Increased numbers of small vacuoles were observed in the cytoplasm of the PTCs from a 5-year-old TG+ mouse as compared with WT mouse (B, arrow). Increased PAS stain (C, arrow) but no increased ORO staining (D) was observed in the cytoplasm of the TG+ kidney cells. All proximal tubules stained by PAS were abnormal proximal tubules with "clear" cells (D). All scale bars represent 100 μm.
ccRCC are either not changed (P < 0.05) or are not significantly upregulated (< 3 fold) in the γ-HIF2αM3 TG+ compared with WT kidneys (Table 1).

We also identified the top genes overexpressed at the RNA level in the γ-HIF2αM3 TG+ compared with WT kidneys, and compared the transcript levels of these genes with those in the combined Oncomine datasets used in Table 1. One of these genes, CD163 molecule-like 1, shows overexpression (fold change > 3) in the combined Oncomine datasets (Table 2). The other 19 genes that are highly overexpressed in the γ-HIF2αM3 TG+ relative to WT kidneys do not show overexpression in the combined Oncomine human ccRCC versus normal datasets (Table 2). We conclude from analysis of these data that expression of a mutant, constitutively active HIF2α protein in kidney proximal tubules does not result in a transcriptome that closely resembles that of human ccRCC.

**Kidneys from γ-HIF1αM3(TRACK); γ-HIF2αM3 double TG+ mice are similar to kidneys from γ-HIF1αM3 TG+ mice**

We also examined kidneys from 8 γ-HIF1αM3; γ-HIF2αM3 double TG+ male mice (from 6- to 11-months-old). Both HIF1αM3 and HIF2αM3 transgenes are expressed in the kidneys from the γ-HIF1αM3; γ-HIF2αM3 double TG+ mice as detected by RT-PCR using transgene-specific primers (Fig. 5A). We did not detect CD70 transcripts in these double TG+ kidneys (Fig. 5A), but we did detect CT70 transcripts in the kidneys of 15-month-old TRACK kidneys, as reported (13). Seven of 8 γ-HIF1αM3; γ-HIF2αM3 TG+ mice did not show any greater tumorigenesis in the kidney cortex than the γ-HIF1αM3 single TG+ TRACK mice. One of the double TG+ mice had kidneys with a few disorganized proximal tubules (Fig. 5B), resembling those observed in TRACK mice ages 14 months or older (13). Because 7 of the 8 γ-HIF1αM3; γ-HIF2αM3 double TG+ mice showed no pathologic features indicative of greater tumorigenicity than that in the TRACK mice, which express only a mutated, constitutively active form of HIF1α, we conclude that a mutated, constitutively active form of HIF2α does not promote tumorigenesis in the presence of a constitutively active HIF1α in the kidney proximal tubules.

**Discussion**

**The γ-HIF2αM3 TG+ kidneys do not resemble human ccRCC histologically**

Several researchers have suggested that overexpression of HIF2α is a key step in the development of ccRCC (4). However, we detected no obvious ccRCC phenotype in the kidneys of γ-HIF2αM3 TG+ mice. In contrast, in our γ-HIF1α M3 (TRACK) transgenic mice, we identified 2 types of vacuolation: large round, discrete vacuoles, displacing the nucleus, that contained lipid accumulation (13); and vacuoles with a pale, eosinophilic to clear featherly cytoplasm without displacement of the nucleus, consistent with glycogen accumulation and hydropic degeneration (13). Although some PTCs of these γ-HIF2αM3 transgenic mice contain clear spaces around the nuclei, the vacuoles do not resemble the large, almost completely clear vacuoles found in the TRACK kidneys (13) and in human ccRCC (38). Furthermore, no renal cysts or disorganized proximal tubules resembling in situ carcinoma were identified in any of the 37 TG+ animals examined, including 6 between 24- and 33-months-old. These data suggest that overexpression of HIF2α alone in PTCs does not result in PTCs that resemble ccRCC histologically.

**The γ-HIF2αM3 TG+ kidneys do not show neoplastic transformation and do not express cancer markers**

That there is no ccRCC phenotype in these γ-HIF2αM3 TG+ kidneys is unexpected, given previous data in the literature using cultured human ccRCC cells (9, 12, 32, 33, 39). HIF2α has previously been reported to be more oncogenic than HIF1α and to play an important role in ccRCC carcinogenesis (4, 40, 41). However, we detected no renal cysts or neoplasia in the kidneys of γ-HIF2αM3 TG+ mice. In contrast, we observed multiple renal cysts and disorganized proximal tubules resembling in situ carcinoma in the γ-HIF1α M3 TG+ TRACK mice (13). Uncontrolled cell division/proliferation and genomic instability are two of the most prominent features of tumor cells. By staining, using Ki67 or γH2AX antibodies, we previously showed elevated expression of H2AX in the kidneys by RT-PCR (Fig. 2), again indicating a lack of neoplastic transformation.

Using the methods described in the supplementary data, we calculated and compared the relative levels of the HIF1αM3 and HIF2αM3 transcripts in the HIF1αM3 TG+ (TRACK) mice...
<table>
<thead>
<tr>
<th>Oncomine median rank</th>
<th>Oncomine median-fold change</th>
<th>Gene</th>
<th>Gene description</th>
<th>Fold-change $\gamma$-HIF2α-M3/WT</th>
<th>Student t test-BH-FDR-q-value</th>
<th>Fisher exact-R $\gamma$-HIF2α-M3/WT-BH-FDR-q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>5.55E-23</td>
<td>NDUFA4L2</td>
<td>NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2</td>
<td>1.7</td>
<td>0.049</td>
<td>1.95E-016</td>
</tr>
<tr>
<td>14</td>
<td>3.91E-13</td>
<td>C7orf88</td>
<td>chromosome 7 open reading frame 68, or hypoxia inducible gene 2</td>
<td>1.4</td>
<td>0.071</td>
<td>0.005</td>
</tr>
<tr>
<td>14</td>
<td>2.82E-11</td>
<td>ENO2</td>
<td>enolase 2, gamma neuronal</td>
<td>1.0</td>
<td>0.816</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>9.50E-19</td>
<td>EGLN3</td>
<td>EGL 9 homolog 3 (Caenorhabditis elegans)</td>
<td>3.6</td>
<td>0.007</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>1.73E-14</td>
<td>IGFBP3</td>
<td>insulin-like growth factor binding protein 3</td>
<td>1.7</td>
<td>0.054</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>1.23E-10</td>
<td>SPAG4</td>
<td>sperm-associated antigen 4</td>
<td>1.3</td>
<td>0.571</td>
<td>0.789</td>
</tr>
<tr>
<td>28</td>
<td>2.83E-17</td>
<td>AHNAK2</td>
<td>AHNAK nucleoprotein 2</td>
<td>1.5</td>
<td>0.518</td>
<td>0.071</td>
</tr>
<tr>
<td>30</td>
<td>3.79E-7</td>
<td>TMCC1</td>
<td>transmembrane and coiled coil domains 1</td>
<td>1.1</td>
<td>0.199</td>
<td>0.002</td>
</tr>
<tr>
<td>36</td>
<td>2.19E-16</td>
<td>RNASET2</td>
<td>ribonuclease T2A; RNASET2</td>
<td>1.1</td>
<td>0.934</td>
<td>0.531</td>
</tr>
<tr>
<td>38</td>
<td>1.93E-13</td>
<td>CAV1</td>
<td>caveolin 1, caveolae protein</td>
<td>1.1</td>
<td>0.038</td>
<td>1.33E-036</td>
</tr>
<tr>
<td>41</td>
<td>2.54E-13</td>
<td>SLC16A3</td>
<td>solute carrier family 16 (monocarboxylic acid transporters), member 3</td>
<td>0.8</td>
<td>0.114</td>
<td>0.002</td>
</tr>
<tr>
<td>46</td>
<td>6.8E-10</td>
<td>LCPII</td>
<td>lymphocyte cytosolic protein 2</td>
<td>1.2</td>
<td>0.600</td>
<td>0.161</td>
</tr>
<tr>
<td>47</td>
<td>6.92E-16</td>
<td>CA-IX</td>
<td>carbonic anhydrase</td>
<td>1.9</td>
<td>0.032</td>
<td>7.13E-093</td>
</tr>
<tr>
<td>48</td>
<td>7.84E-10</td>
<td>NETO2</td>
<td>neuropilin (NRP) and tolloid (TLL)-like 2</td>
<td>1.3</td>
<td>0.539</td>
<td>0.299</td>
</tr>
<tr>
<td>49</td>
<td>8.25E-10</td>
<td>IFNGR2</td>
<td>IFN 3 receptor 2</td>
<td>1.2</td>
<td>0.113</td>
<td>4.71E-016</td>
</tr>
<tr>
<td>57</td>
<td>3.12E-15</td>
<td>ABCG1</td>
<td>ATP-binding cassette, subfamily G (WHITE), member 1</td>
<td>1.4</td>
<td>0.185</td>
<td>7.39E-012</td>
</tr>
<tr>
<td>57</td>
<td>1.37E-12</td>
<td>UBE2L6</td>
<td>ubiquitin-conjugating enzyme E2L 6</td>
<td>1.0</td>
<td>0.902</td>
<td>0.978</td>
</tr>
<tr>
<td>59</td>
<td>1.59E-10</td>
<td>DIAPH2</td>
<td>diaphanous homolog 2 (Drosophila)</td>
<td>1.1</td>
<td>0.329</td>
<td>0.003</td>
</tr>
<tr>
<td>59</td>
<td>1.50E-9</td>
<td>ALDOA</td>
<td>aldolase A, fructose-bisphosphate</td>
<td>1.0</td>
<td>0.759</td>
<td>4.91E-005</td>
</tr>
<tr>
<td>59</td>
<td>1.69E-8</td>
<td>SLC15A4</td>
<td>solute carrier family 15, member 4</td>
<td>1.0</td>
<td>0.651</td>
<td>0.108</td>
</tr>
</tbody>
</table>

**NOTE:** The list of the top 20 genes overexpressed at the RNA level in human ccRCC was compiled by Oncomine by combining 5 different datasets of human ccRCC patient samples, totaling 175 patients. These 5 datasets are (i) hereditary ccRCC versus normal (32), (ii) nonhereditary ccRCC versus normal (32), (iii) ccRCC versus normal (33), (iv) ccRCC versus normal (34), and (v) ccRCC versus normal (35). The fold changes in mRNA levels of these genes in $\gamma$-HIF2αM3 TG + mice versus WT kidneys are listed.

*aOne of these genes (EGLN3) shows statistically significant ($P < 0.05$) overexpression (fold change > 3) in $\gamma$-HIF2αM3 TG + versus WT mice.*
Table 2. Top 20 genes overexpressed at the mRNA level in the γ-HIF2α-M3 TG+ versus WT kidneys by RNAseq

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>121.37</td>
<td>0</td>
<td>0</td>
<td>ENSMUSG00000025515</td>
<td>mucin 2</td>
<td>-1.09</td>
</tr>
<tr>
<td>57.1</td>
<td>4.21E-006</td>
<td>4.46E-147</td>
<td>ENSMUSG00000034486</td>
<td>gastrulation brain homeobox 2</td>
<td>-1.02</td>
</tr>
<tr>
<td>48.32</td>
<td>0</td>
<td>0</td>
<td>ENSMUSG00000003111</td>
<td>α-2-macroglobulin</td>
<td>1.81</td>
</tr>
<tr>
<td>45.86</td>
<td>2.03E-005</td>
<td>7.48E-083</td>
<td>ENSMUSG00000026468</td>
<td>LIM homeobox protein 4</td>
<td>1.77</td>
</tr>
<tr>
<td>36.31</td>
<td>0</td>
<td>0</td>
<td>ENSMUSG00000050645</td>
<td>defensin β 19</td>
<td>0.38</td>
</tr>
<tr>
<td>33.13</td>
<td>2.03E-005</td>
<td>0</td>
<td>ENSMUSG00000032080</td>
<td>apolipoprotein A-N</td>
<td>-1.23</td>
</tr>
<tr>
<td>21.74</td>
<td>0.01</td>
<td>0</td>
<td>ENSMUSG00000056296</td>
<td>synaptoporin</td>
<td>-1.15</td>
</tr>
<tr>
<td>19.73</td>
<td>1.71E-144</td>
<td>0</td>
<td>ENSMUSG00000024008</td>
<td>copine V; similar to Copine V</td>
<td>1.54</td>
</tr>
<tr>
<td>19.15</td>
<td>2.02E-042</td>
<td>0</td>
<td>ENSMUSG00000018893</td>
<td>myoglobin</td>
<td>-1.09</td>
</tr>
<tr>
<td>17.16</td>
<td>1.27E-005</td>
<td>0</td>
<td>ENSMUSG00000032081</td>
<td>apolipoprotein C-III</td>
<td>-1.7</td>
</tr>
<tr>
<td>17.14</td>
<td>0</td>
<td>0</td>
<td>ENSMUSG00000021708</td>
<td>RAS protein-specific guanine nucleotide-releasing factor 2</td>
<td>1.57</td>
</tr>
<tr>
<td>15.5</td>
<td>0.02</td>
<td>5.83E-090</td>
<td>ENSMUSG0000000731</td>
<td>autoimmune regulator (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy)</td>
<td>-1.08</td>
</tr>
<tr>
<td>14.24</td>
<td>0.03</td>
<td>1.05E-033</td>
<td>ENSMUSG00000042288</td>
<td>solute carrier family 26, member 9</td>
<td>-0.23</td>
</tr>
<tr>
<td>10.97</td>
<td>6.47E-005</td>
<td>9.41E-094</td>
<td>ENSMUSG00000025461</td>
<td>CD163 molecule-like 1α</td>
<td>3.15</td>
</tr>
<tr>
<td>7.49</td>
<td>0.01</td>
<td>0</td>
<td>ENSMUSG00000038418</td>
<td>early growth response 1</td>
<td>-1.16</td>
</tr>
<tr>
<td>6.49</td>
<td>1.72E-038</td>
<td>0</td>
<td>ENSMUSG00000030236</td>
<td>solute carrier organic anion</td>
<td>-1.07</td>
</tr>
<tr>
<td>6.26</td>
<td>1.01</td>
<td>6.27E-010</td>
<td>ENSMUSG00000052468</td>
<td>peripheral myelin protein 2</td>
<td>-1.14</td>
</tr>
<tr>
<td>6.14</td>
<td>1.01</td>
<td>2.65E-009</td>
<td>ENSMUSG00000054667</td>
<td>insulin receptor substrate 4; similar to insulin receptor substrate 4</td>
<td>-1.16</td>
</tr>
<tr>
<td>5.62</td>
<td>0.02</td>
<td>3.81E-011</td>
<td>ENSMUSG00000026959</td>
<td>glutamate receptor, ionotropic, NMAD1 (ζ1)</td>
<td>1.34</td>
</tr>
<tr>
<td>5.39</td>
<td>0.05</td>
<td>1.46E-271</td>
<td>ENSMUSG00000024411</td>
<td>aquaporin 4</td>
<td>-1.01</td>
</tr>
</tbody>
</table>

NOTE: The list of the top 20 genes overexpressed at the mRNA level in the γ-HIF2α-M3 TG+ versus WT kidneys was compiled from the RNAseq results. The median fold change in mRNA levels of these genes in human ccRCC was compiled by Oncomine by combining 5 different sets of human ccRCC patient samples, totaling 175 patients (see Table 1). Genes that have no measurements from all 5 datasets were excluded from this list.

*aOne of these genes (Cd163l1) shows overexpression (fold change > 3) in the Oncomine datasets.
ccRCC patient samples showed statistically significant overexpression in the top 20 genes overexpressed in 5 different sets of human ccRCC Oncomine datasets. These data further indicate that expression of mutated, constitutively active HIF2α in kidney PTCs does not lead to a phenotype resembling human ccRCC, though some HIF2α target genes, such as CA-IX, show increased expression in the PTCs of kidneys of γ-HIF2α M3 TG+ mice (Fig. 2). We did not detect high levels of the putative HIF2α targets TGFα, cyclin D1, and Oct4 (42, 43) in the HIF2α M3 TG+ mice. This might be because different types of cells were used in those assays. For example, Oct4 was identified as a HIF2α target in embryonic stem cells (42), whereas TGFα and cyclin D1 were identified as HIF2α targets in cultured ccRCC cancer cells (43).

### Expression of constitutively active HIF2α does not cause neoplastic transformation of proximal tubule cells

Our data suggest that expression of a mutated, constitutively active HIF2α in proximal tubule cells, the cells from which ccRCC originates (44, 45), does not cause neoplastic transformation. This conclusion is also supported by a recent publication reporting that overexpression of a similarly mutated (P405A, P530G, and N851A), constitutively active HIF2α in murine distal tubule cells is insufficient for renal carcinogenesis (16). However, it is possible that HIF2α may cooperate with HIF1α to drive renal cell carcinogenesis, because an increase in HIF2α expression is seen after an increase in HIF1α expression in early kidney lesions in patients with VHL disease (46). We crossed our TRACK and γ-HIF2α M3 TG+ mice to determine whether constitutive activation of both HIF1α and HIF2α results in more rapid neoplastic transformation of normal PTCs. From pathologic examination of 8 γ-HIF1α M3; γ-HIF2α M3 TG+ mice up to 11 months old, we conclude that constitutively active HIF2α does not result in more rapid neoplastic transformation in the presence of constitutively active HIF1α in the kidney. However, we can not rule out the possibility that this lack of tumorigenicity of HIF2α M3 is caused by potential biologic differences between human and mouse kidney PTCs.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors’ Contributions

Conception and design: L. Fu, M.M. Shevchuk, D.M. Nanus, L.J. Gudas

Development of methodology: L. Fu, L.J. Gudas

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Fu, G. Wang, M.M. Shevchuk, L.J. Gudas

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Fu, G. Wang, M.M. Shevchuk, D.M. Nanus, L.J. Gudas

Writing, review, and/or revision of the manuscript: L. Fu, M.M. Shevchuk, D.M. Nanus, L.J. Gudas

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.J. Gudas

Study supervision: L.J. Gudas

### Acknowledgments

The authors thank Dr. Simon for the WT HIF2α construct, Dr. Campagne for the help with GobyWeb software, and Dr. Yuo Zhang (Genomics Resources Core Facility of WCMC) for help with quantification of GGT-HIF2α M3 data.

---

![Image](image_url)
Grant Support
This work was supported by WCMC, the Turobiner Kidney Cancer Research Fund, and the Genitourinary Oncology Research Fund. Drs. L. Fu holds the Robert H. McCooey Genitourinary Oncology Research Fellowship and G. Wang was partially supported by the Nutrition and Cancer Prevention R25 Training Grant (NCIR25105012). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

17. Medina Villamil V, Aparicio Gallego G, Santamarina Caiznos I, Valla-
18. Biswas S, Charlesworth PJ, Turner GD, Leek R, Thambor PT, Campo L, et al. CD31 angiogenesis and combined expression of HIF-1alpha and HIF-2alpha are prognostic in primary clear-cell renal cell carcinoma (CC-RCC), but HIFalpha transcriptional products are not implic-
ations for antiangiogenic trials and HIFalpha biomarker studies in primary CC-RCC. Carcinogenesis 2012;33:1717–25.
32. Alt JR, Cleveland JL, Hannirik M, Diewi JA. Phosphoprotein-depend-

Fu et al.

Cancer Res; 73(9) May 1, 2013


Activation of HIF2α in Kidney Proximal Tubule Cells Causes Abnormal Glycogen Deposition but not Tumorigenesis

Leiping Fu, Gang Wang, Maria M. Shevchuk, et al.

Cancer Res 2013;73:2916-2925. Published OnlineFirst February 27, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-3983

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/02/27/0008-5472.CAN-12-3983.DC1

Cited articles
This article cites 45 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/9/2916.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/73/9/2916.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/73/9/2916.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.