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Activation of HIF2 α in Kidney Proximal Tubule Cells Causes Abnormal Glycogen Deposition but Not Tumorigenesis

Leiping Fu^{1,4}, Gang Wang^{1,4}, Maria M. Shevchuk^{2,4}, David M. Nanus^{3,4}, and Lorraine J. Gudas^{1,4,*}

¹Department of Pharmacology, Weill Cornell Medical College (WCMC) of Cornell University, New York, NY 10065, USA

²Department of Pathology and Laboratory Medicine, Weill Cornell Medical College (WCMC) of Cornell University, New York, NY 10065, USA

³Division of Hematology and Medical Oncology of the Department of Medicine, Weill Cornell Medical College (WCMC) of Cornell University, New York, NY 10065, USA

⁴Weill Cornell Cancer Center, Weill Cornell Medical College (WCMC) of Cornell University, New York, NY 10065, USA

Abstract

Renal cell carcinoma (RCC) is the most common primary cancer arising from the kidney in adults, with clear cell carcinoma (ccRCC) representing ~75% of all RCCs. Increased expression of the hypoxia-induced factors HIF1 α and HIF2 α 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 HIF2 α (P405A, P530A and N851A, HIF2 α M3) in the proximal tubules of mice is not sufficient to promote ccRCC by itself, nor does it enhance HIF1 α M3 oncogenesis when co-expressed with constitutively active HIF1 α M3. Neoplastic transformation in kidneys was not detected at up to 33 months of age, nor was increased expression of Ki67 (MKI67), γ H2AX (H2AFX) or CD70 observed. Further, the genome-wide transcriptome of the transgenic kidneys does not resemble human ccRCC. We conclude that a constitutively active HIF2 α is not sufficient to cause neoplastic transformation of proximal tubules, arguing against the idea that HIF2 α activation is critical for ccRCC tumorigenesis.

Keywords

clear cell renal cell carcinoma; murine cancer model; kidney cancer; RNA-seq

Introduction

Renal cell carcinoma (RCC) is the most common primary cancer arising from the kidney in adults, with clear cell carcinoma (ccRCC) representing ~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 etiological 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

* To whom correspondence should be addressed: Department of Pharmacology, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065, USA. Tel: (212)746-6250; Fax: (212)746-8858; ljgudas@med.cornell.edu.

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VHL loss is the increased expression of the alpha subunits of hypoxia inducible factors 1 (HIF1 α) and 2 (HIF2 α) (4-6). Increased expression of these two transcription factors has been proposed as a key step in ccRCC carcinogenesis (4). HIF2 α shares ~48% amino acid homology with HIF1 α (7). Both HIF1 α and HIF2 α are regulated by prolyl hydroxylation at proline sites and by asparaginyl hydroxylation at an asparagine site under normoxic conditions (7), and each pairs with HIF1 β and binds to Hypoxia Responsive Elements (HREs, 5'-RCGTG-3') (7). HIF2 α protein levels are increased and HIF2 α is transcriptionally activated in VHL^{-/-}renal carcinomas (8). Furthermore, elimination of HIF2 α in RCC cell lines is sufficient to suppress VHL^{-/-}tumor growth in xenograft models (9, 10). Tumor suppression by pVHL can be overridden by HIF2 α but not HIF1 α in tumor xenografts of cultured 786-0 cells (9, 11, 12). These data collectively suggest that HIF2 α may be more oncogenic than HIF1 α for tumor xenograft growth, although other data indicate that HIF1 α 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 HIF1 α construct (13). We have now employed the same kidney proximal tubule specific type 1 γ -glutamyl transpeptidase (GGT or γ GT) promoter (20, 21) to drive expression of a triple mutant (P405A, P530A and N851A), constitutively active HIF2 α protein specifically in the proximal tubule cells (PTCs) to determine if this results in carcinogenesis. We show here that this triple mutant, HIF2 α protein (HIF2 α M3) is active, even under normoxic conditions. Transgenic mice that express this triple mutant, constitutively active HIF2 α 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, e.g. Ki67, γ H2AX, and CD70, in the kidneys of HIF2 α M3 transgenic positive (TG+) mice. Furthermore, we analyzed entire transcriptomes of cells from the HIF2 α M3 TG+ kidney cortex by Next Generation Sequencing/RNA-seq. The kidney cortex transcriptome of HIF2 α M3 TG+ mice does not closely resemble that of human ccRCC, consistent with the lack of tumorigenesis in these mice.

Material and Methods

Plasmid Construction and Generation of Transgenic Mice

Mutated, constitutively active mouse HIF2 α cDNA was created by site-directed mutagenesis (Invitrogen, Carlsbad, CA) of conserved proline residues (proline 405, 530) and a conserved asparagine (asparagine 851) into alanine residues. The rat GGT promoter (-1930- +246) was amplified by PCR from a plasmid (21). The GGT promoter, mutated HIF2 α , and beta-globin poly-A were cloned into pBlueScript and named γ GT-HIF2 α triple mutant (γ -HIF2 α M3). A linearized XhoI-XbaI fragment (vector sequence removed) was injected into pronuclei of one-cell embryos (C57BL/6 $\times$ C57BL/6) at the WCMC Mouse Genetics Core. Southern Blot analysis was then performed (13). The γ -HIF2 α M3 transgene was carried in the heterozygous state. The γ -HIF2 α M3-1 and the γ -HIF2 α M3-17 lines were mated with the TRACK mice to obtain γ -HIF1 α M3; γ -HIF2 α M3 double TG+ mice. Both γ -HIF1 α M3 and γ -HIF2 α M3 transgenes are carried in the heterozygous state in the double TG+ mice. All animal procedures were performed following guidelines of Research Animal Resource Center.

Tissue Dissection, Processing, Pathological Review, and Histology/Staining

Tissues were fixed, processed, sectioned, and H&E 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 performed as described (13). Antibodies used: HIF2 α (100-122, Novus Biologicals); CA-IX (sc-25600, Santa Cruz); Glut-1 (ab14683, Abcam, Cambridge, MA); Ki67 (M7249, Dako, Denmark); and γ H2AX (9718S, Cell Signaling, Danvers, MA). Periodic Acid/Schiff (PAS) stain was performed on paraffin-embedded and cryo-preserved sections (13). Oil red O (ORO) staining was performed as described (13).

Reverse Transcriptase PCR (RT-PCR), Whole Genome RNA Sequencing, and Data Analysis

Total RNA was extracted using mini-RNAeasy columns (Qiagen, Valencia, CA). Semi-quantitative RT-PCR was then performed (13). Total RNA from thin, outer slices of kidney cortex were used for whole genome sequencing. The complete transcriptomes of kidney cortex from 3 γ -HIF2 α M3 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) (22) in GobyWeb software (23). Comparisons of gene expression changes between γ -HIF2 α M3 TG+ and WT male mice were performed using Differential Expression Analysis with Goby in the GobyWeb. Benjamini and Hochberg FDR adjustment (q-value) for t-test (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 will be deposited in NIH databases upon acceptance for publication.

Statistical Analysis

Results are expressed as the mean $\pm$ SEM. Student's t test was used to determine the statistical significance of the γ H2AX+ and Ki67+ cell number differences between TG+ and WT kidneys.

Results

Generation of transgenic mice that express mutated, constitutively active HIF2 μ

To examine the role of HIF2 μ in ccRCC carcinogenesis we constructed a GGT-HIF2 μ triple mutant plasmid (γ -HIF2 μ M3, Fig. 1A). After confirmation of activity in a cultured normal kidney proximal tubule cell line, a linearized γ -HIF2 μ M3 XbaI-XhoI fragment was used to generate γ -HIF2 μ M3 transgenic mice. A total of 10 out 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 HIF2 μ (γ -HIF2 μ M3) 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 HIF1 μ , and endogenous HIF2 μ mRNA levels were not changed in the kidneys of TG+ mice compared with the kidneys of transgenic negative (TG-) mice (Fig. 1C, TG-). All five TG+ founder lines, γ -HIF2 μ M3-1, γ -HIF2 μ M3-17, γ -HIF2 μ M3-27, γ -HIF2 μ M3-31 and γ -HIF2 μ M3-48, developed normally and could pass the transgene to offspring following a Mendelian pattern of inheritance. Since 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.

HIF2 μ and its target genes are upregulated in TG+ kidneys

As described above, the HIF2 μ transgene mRNA is expressed in TG+ kidneys (Fig. 1C). We next examined the expression by semi-quantitative RT-PCR of CA-IX (NP_647466) and Glut-1 (NP_035530), which are HIF2 μ target genes, and CD70 (TNFSF7), which is a marker of human ccRCC (24-27) but not a known HIF2 μ target gene. We detected increased mRNA levels of CA-IX and Glut-1, but not CD70 (Fig. 2A).

By immunohistochemistry, we confirmed increased HIF2 μ , CA-IX, and Glut1 protein staining in the abnormal proximal tubules (PTs) (Fig. 2B, C, D, TG+), but not in the morphologically normal PTs of the same γ -HIF2 μ M3 mice. As expected, weak or no detectable HIF2 μ , CA-IX, or Glut1 signals are observed in the PTs of TG- and WT mice (Fig. 2B, C, D, TG-).

γ -HIF2 μ M3-1 and γ -HIF2 μ M3-17 TG+ mice exhibit mild vacuolation

Clear vacuoles were detected circumferentially around some, but not all, proximal tubule cell nuclei in the γ -HIF2 μ M3 mice. The γ -HIF2 μ M3-1 and γ -HIF2 μ M3-17 lines exhibited the strongest phenotype (Fig. 3A); the other lines had similar phenotypes, but displayed fewer PTs 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 -HIF2 μ M3-1 and γ -HIF2 μ M3-17 mice. Increased numbers of vacuoles in some proximal tubules were observed in two year old γ -HIF2 μ M3-1 and γ -HIF2 μ M3-17 mice (Fig. 3B, arrows). The histologically abnormal PT cells in TG+ mice were large, simple cuboidal epithelial cells (Fig. 3A and 3B) 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 HIF2 μ TG+ mice contained increased amounts of glycogen, as demonstrated by Periodic Acid/Schiff (PAS) staining (Fig. 3C, arrows), but no increase in lipid in as determined by Oil Red O (ORO) staining (Fig. 3D).

We examined 21 mice of the γ -HIF2 μ M3-1 line and 16 γ -HIF2 μ M3-17 TG+ mice and compared them to 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 -HIF2 μ M3 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 serine 139 phosphorylated form of H2A histone family, member X (γ H2AX) is a widely used marker that indicates double strand breaks (DSBs). We examined Ki67 and γ H2AX protein levels in TG+ vs. TG-kidneys (Fig. 4). We detected few Ki67+ and γ H2AX+ cells in WT PTs (1.5 or 1.9 TG- cells/field, respectively). In the γ -HIF2 μ M3 TG+ mice we observed statistically significantly decreased numbers of Ki67+ and γ H2AX+ cells in the morphologically abnormal PTs. 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 -HIF2 μ M3-1 and γ -HIF2 μ M3-17 TG+ kidneys, respectively; there were 0.9 ($P=0.03$, compared with TG-) and 0.8 ($P=0.005$, compared with TG-) γ H2AX+ cells/field in the γ -HIF2 μ M3-1 and γ -HIF2 μ M3-17 TG+ kidneys, respectively. Thus, the kidneys from TG+ mice exhibited a *reduction* in Ki67 and γ H2AX staining relative to WT.

The transcriptome of γ -HIF2 μ M3 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, e.g. GGT, indicate kidney cortex identity.

We identified upregulation of 1253 and downregulation of 749 transcripts in γ -HIF2 μ M3 TG+ vs WT kidney cortices from 18 month old mice using less stringent conditions (fold change >1.2 , 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 -HIF2 μ M3 TG+ vs. WT mice when data were filtered with a more stringent condition (fold change >1.5 , t-test-BH-FDR-q-value <0.01 , fisher-exact-test-BH-FDR-q-value <0.01). Certain HIF2 μ target genes, such as CA-IX, show significant overexpression in the HIF2 μ M3 TG+ vs. WT samples. However, other putative HIF2 μ target genes, such as Oct4, cyclin D1, and TGF β (32, 33), did not exhibit increased expression in the γ -HIF2 μ M3 TG+ vs. WT kidneys. Cyclin D1 shows a fold change of 1.1 (t-test-BH-FDR-q-value =0.49) and TGF β transcripts show a fold change of 1.24, (t-test-BH-FDR-q-value =0.19).

To compare the transcriptome of the γ -HIF2 μ M3 TG+ kidneys to human ccRCC, we identified the 20 genes most highly overexpressed at the RNA level in human ccRCC from Oncomine (Compendia Bioscience, Ann Arbor, MI), combining five different datasets of human ccRCC patient samples (34-37). We identified a total of 5 datasets of Cancer vs. Normal Analysis of the ccRCC cancer type (Table 1). The five datasets we utilized are in Table 1. One of the 20 genes highly expressed in human ccRCC, EGLN3, shows significant upregulation in our γ -HIF2 μ M3 TG+ kidneys (>3 fold) (Table 1). However, the remaining 19 genes that are highly overexpressed in human ccRCC are either not changed ($p<0.05$) or are not significantly upregulated (<3 fold) in the γ -HIF2 μ M3 TG+ compared to WT kidneys (Table 1).

We also identified the top genes overexpressed at the RNA level in the γ -HIF2 μ M3 TG+ compared to WT kidneys, and compared the transcript levels of these genes to 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 -HIF2 μ M3 TG+ relative to WT kidneys do not show overexpression in the combined Oncomine human ccRCC vs. normal datasets (Table 2). We conclude from analysis of these data that expression of a mutant, constitutively active HIF2 μ protein in kidney PTs does not result in a transcriptome that closely resembles that of human ccRCC.

Kidneys from γ -HIF1 μ M3(TRACK); γ -HIF2 μ M3 double TG+ mice are similar to kidneys from γ -HIF1 μ M3 TG+ mice

We also examined kidneys from 8 γ -HIF1 μ M3; γ -HIF2 μ M3 double TG+ male mice (from 6 to 11 months old). Both HIF1 μ M3 and HIF2 μ M3 transgenes are expressed in the kidneys from the γ -HIF1 μ M3; γ -HIF2 μ M3 double TG+, 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 CD70 transcripts in the kidneys of 15 month old TRACK kidneys, as reported (13). Seven of eight γ -HIF1 μ M3; γ -HIF2 μ M3 TG+ mice did not show any greater tumorigenesis in the kidney cortex than the γ -HIF1 μ M3 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 aged 14 months or older (13). Since seven of the eight γ -HIF1 μ M3; γ -HIF2 μ M3 double TG+ mice showed no pathological features indicative of greater tumorigenicity than that in the TRACK mice, which express only a mutated, constitutively active form of HIF1 μ , we conclude that a mutated, constitutively active form of HIF2 μ does not promote tumorigenesis in the presence of a constitutively active HIF1 μ in the kidney proximal tubules.

Discussion

The γ -HIF2 μ M3 TG+ kidneys do not resemble human ccRCC histologically

Several researchers have suggested that overexpression of HIF2 μ is a key step in the development of ccRCC (4). However, we detected no obvious ccRCC phenotype in the kidneys of γ -HIF2 μ M3 TG+ mice. In contrast, in our γ -HIF1 μ -M3 (TRACK) transgenic mice we identified two types of vacuolation: large round, discrete vacuoles, displacing the nucleus, that contained lipid accumulation (13); and vacuoles with a pale, eosinophilic to clear feathery cytoplasm without displacement of the nucleus, consistent with glycogen accumulation and hydropic degeneration (13). Although some PTCs of these γ -HIF2 μ M3 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 PTs resembling *in situ* carcinoma were identified in any of the 37 TG+ animals examined, including 6 between 24 to 33 months old. These data suggest that overexpression of HIF2 μ alone in PT cells does not resemble ccRCC histologically.

The γ -HIF2 μ M3 TG+ kidneys do not show neoplastic transformation and do not express cancer markers

That there is no ccRCC phenotype in these γ -HIF2 μ M3 TG+ kidneys is unexpected given previous data in the literature using cultured human ccRCC cells (9-12, 32, 33, 39). HIF2 μ has previously been reported to be more oncogenic than HIF1 μ 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 -HIF2 μ M3 TG+ mice. In contrast, we observed multiple renal cysts and disorganized PTs resembling *in situ* carcinoma in the γ -HIF1 μ -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 γ H2AX antibodies, we previously showed elevated expression of both Ki67 and γ H2AX in γ -HIF1 μ -M3 TG+ mice (13). However, we did not detect higher levels of Ki67 or γ H2AX in the γ -HIF2 μ M3 TG+ kidneys (Fig. 4). In fact, the levels were lower than in WT, suggesting that constitutively active HIF2 μ *reduces* cell proliferation and genomic instability. The CD70 protein level is higher in the majority of human ccRCC and CD70 is a marker and therapeutic target in human ccRCC (24). CD70 mRNA levels are several fold higher in older TRACK vs WT mice (13). However, we didn't observe any CD70 mRNA expression in 18 month old γ -HIF2 μ M3 TG+ kidneys by RT-PCR (Fig. 2), again indicating a lack of neoplastic transformation.

Using the methods described in the supplemental data, we calculated and compared the relative levels of the HIF1 μ M3 and HIF2 μ M3 transcripts in the HIF1 μ M3 TG+ (TRACK) mice and HIF2 μ M3 TG+ mice, respectively. The HIF2 μ M3 mRNA levels in the HIF2 μ M3

TG+ mice are about 26% of the HIF1 μ M3 mRNA levels in the highest expressing TRACK line, and about the same as the HIF1 μ M3 mRNA levels in other TRACK transgenic lines (supplemental Table S2). The HIF2 μ M3 protein is also overexpressed in the HIF2 μ M3 kidneys (Fig. 2B). Thus, the difference we observe in tumorigenic phenotype is likely a result of the specific HIF protein expressed and not the level of transgene expression.

The transcriptome of γ -HIF2 μ M3 TG+ kidneys does not closely resemble the transcriptome of human ccRCC

Furthermore, we compared the changes in gene expression in the γ -HIF2 μ M3 TG+ kidneys to human ccRCC. Only one of the top 20 genes overexpressed in five different sets of human ccRCC patient samples showed statistically significant overexpression in the γ -HIF2 μ M3 TG+ kidneys (Table 1). In fact, some of these genes showed a statistically significant *reduction* in expression, e.g. caveolin 1 showed a fold change of 0.79 (t-test-BH-FDR-q-value = 0.04) when we compared the γ -HIF2 μ M3 TG+ to WT kidneys. Conversely, only one of the top 20 genes overexpressed at the RNA level in the γ -HIF2 μ M3 TG+ kidneys showed overexpression in the combined human ccRCC Oncomine datasets (Table 2). These data further indicate that expression of mutated, constitutively active HIF2 μ in kidney PTCs does not lead to a phenotype resembling human ccRCC, though some HIF2 μ target genes, such as CA-IX, show increased expression in the PTCs of kidneys of γ -HIF2 μ M3 TG+ mice (Fig. 2). We did not detect high levels of the putative HIF2 μ targets TGF μ , cyclin D1, and Oct4 (42, 43) in the HIF2 μ M3 TG+mice. This might be because different types of cells were used in those assays. For example, Oct4 was identified as a HIF2 μ target in embryonic stem cells (42), while TGF μ and cyclin D1 were identified as HIF2 μ targets in cultured ccRCC cancer cells (43).

Expression of constitutively active HIF2 μ does not cause neoplastic transformation of proximal tubule cells

Our data suggest that expression of a mutated, constitutively active HIF2 μ 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, N851A), constitutively active HIF2 μ in murine *distal* tubule cells is insufficient for renal carcinogenesis (16). However, it is possible that HIF2 μ may cooperate with HIF1 μ to drive renal cell carcinogenesis, since an increase in HIF2 μ expression is seen *after* an increase in HIF1 μ expression in early kidney lesions in VHL disease patients (46). We crossed our TRACK and γ -HIF2 μ M3 TG+ mice to determine if constitutive activation of both HIF1 μ and HIF2 μ results in more rapid neoplastic transformation of normal PTCs. From pathological examination of eight γ -HIF1 μ M3; γ -HIF2 μ M3 TG+ mice up to 11 months old, we conclude that constitutively active HIF2 μ does not result in more rapid neoplastic transformation in the presence of constitutively active HIF1 μ in the kidney. However, we can't rule out the possibility that this lack of tumorigenicity of HIF2 μ M3 is caused by potential biological differences between human and mouse kidney PT cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation of γ -HIF2 μ M3 transgenic mice

A. Construction of the γ -HIF2 μ M3 plasmid. Only the fragment used to create the TG+ mice is shown. Three mutations (P405A, P530A and N851A), dashed arrows. An intron (shaded square) is included in the β -globin poly A. Primers 1 and 2 are 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.** HIF2 μ transgene, endogenous VHL, HIF1 μ , and HIF2 μ RT-PCR in kidneys of the γ -HIF2 μ M3-1 line. The HIF2 μ transgene was only detected in the TG+ mice. Endogenous VHL, HIF1 μ , and HIF2 μ mRNAs are expressed at similar levels in TG+ and TG-mice. β -Actin, loading control. **D.** HIF2 μ transgene RT-PCR in multiple organs of the γ -HIF2 μ M3-1 line mice. The HIF2 μ transgene is detected specifically in the kidneys and testes of TG+ mice from the γ -HIF2 μ M3-1 line. No expression of the γ -HIF2 μ M3 transgene was detected in any organ of the TG-mouse. β -Actin, loading control.

Figure 2. Overexpression of HIF2μ target genes in the γ-HIF2μM3 kidneys

Increased levels of HIF2μ target genes, e.g. CA-IX, Glut-1, in about 18 month old γ-HIF2μM3 kidney. A. Increased mRNA levels of CA-IX and Glut-1 and no expression of CD70 by semi-quantative RT-PCR. B, C, and D, representative images of the immunohistochemistry staining using HIF2μ (B), CA-IX (C) and Glut-1 (D) antibodies. Increased staining of HIF2μ (B, arrow), CA-IX (C, arrow) and Glut-1 (D, arrow) is only observed in the abnormal PTs of the TG+ mice. All scale bars represent 100 μm.

Figure 3. “Clear” cells in γ -HIF2 μ M3-43 mice

Representative images of the histological morphology (A, B), PAS stain (C), and ORO stain (D) of the “clear” cells observed in γ -HIF2 μ M3-1 and γ -HIF2 μ M3-17 male TG+ and TG- kidneys. Vacuoles with a pale, eosinophilic to clear feathery cytoplasm without displacement of the nucleus are observed in 1 year old TG+ mice as compared to WT mice (A, arrow). Increased numbers of small vacuoles are observed in the cytoplasm of the PT cells from a 2 year old TG+ mouse as compared to WT mouse (B, arrow). Increased PAS stain (C, arrow) but no increased ORO staining (D) is observed in the cytoplasm of the TG+ kidney cells. All PTs stained by PAS were abnormal PTs with “clear” cells (C). All scale bars represent 100 μ m.

Figure 4. Decreased expression of γ H2AX and Ki67 in the γ -HIF2 μ M3 kidneys

The Ki67+ and γ H2AX+ cells in 10 random fields of sections of 3 different TG+ and 3 WT kidneys were counted. All TG+ mice show a statistically significant decrease in Ki67+ and γ H2AX+ cells. * P<0.05, ** P<0.01.

Figure 5. Transgene expression and histopathology in γ -HIF1 μ M3; γ -HIF2 μ M3 double TG+ mice

A. HIF1 μ M3 and HIF2 μ M3 transcripts are expressed in kidneys from double TG+, 11 month mice. CD70 transcripts are not detected in the double TG+ kidneys, but are detected in kidneys from 15 month old HIF1 μ M3-43 kidneys. B. One disorganized proximal tubule in a γ -HIF1 μ M3; γ -HIF2 μ M3 double TG+, 11 month old mouse. Scale bar represents 50 μ m.

Table 1
Twenty genes showing the highest mRNA expression in human ccRCC compared to normal human kidney, from the Oncomine databas

The list of the top 20 genes overexpressed at the RNA level in human ccRCC was retrieved from Oncomine by combining five different datasets of human ccRCC patient samples, totaling 175 patients. These five datasets are 1.) Hereditary ccRCC vs. Normal (32), 2.) Non-Hereditary ccRCC vs. Normal (32), 3.) ccRCC vs. Normal (33), 4.) ccRCC vs. Normal (34), and 5.) ccRCC vs. Normal (35). The fold changes in mRNA levels of these genes in γ -HIF2 α -M3TG+ mice versus WT kidneys are listed. * One of these genes (EGLN3) shows statistically significant ($P < 0.05$) overexpression (fold change > 3) in HIF2 α M3 TG+ vs. WT mice.

Oncomine Median Rank	Oncomine p-Value	Oncomine Median Fold Change	Gene	Gene Description	fold-change γ -HIF2 α -M3/WT	t-test-BH FDR-q-value	fisher-exact-R γ -HIF2 α -M3/WT BH-FDR-q-value
7	5.55E-23	53.9	NDUFA4L2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	1.7	0.049	1.95E-016
14	3.91E-13	16.5	C7orf68	chromosome 7 open reading frame 68, or hypoxia inducible gene 2	1.4	0.071	0.005
14	2.82E-11	12.5	ENO2	enolase 2, gamma neuronal	1.0	0.816	1
16	9.50E-19	12.2	EGLN3	EGL nine homolog 3 (C. elegans)	3.6	0.007	0
21	1.73E-14	11.0	GFBP3	insulin-like growth factor binding protein 3	1.7	0.054	0
25	1.23E-10	12.2	SPAG4	sperm associated antigen 4	1.3	0.571	0.789
28	2.83E-17	15.0	AHNAK2	AHNAK nucleoprotein 2	1.5	0.518	0.071
30	3.79E-7	8.4	TMCC1	transmembrane and coiled coil domains 1	1.1	0.199	0.002
36	2.19E-16	8.6	RNASET2	ribonuclease T2A; RNASET2A	1.1	0.934	0.531
38	1.93E-13	8.2	CAV1	caveolin 1, caveolae protein1	0.8	0.038	1.33E-036
41	2.54E-13	7.1	SLC16A3	solute carrier family 16 (monocarboxylic acid transporters), member 3	0.8	0.114	0.002
46	6.8E-10	4.6	LCP2	lymphocyte cytosolic protein 2	1.2	0.600	0.161
47	6.92E-16	18.0	CA-IX	carbonic anhydrase 9	1.9	0.032	7.13E-093
48	7.84E-10	15.4	NETO2	neuropilin (NRP) and tolloid(TLL)-like 2	1.3	0.539	0.299
49	8.25E-10	2.6	IFNGR2	interferon gamma receptor 2	1.2	0.113	4.71E-016
57	3.12E-15	4.9	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	1.4	0.185	7.39E-012
57	1.37E-12	2.3	UBE2L6	ubiquitin-conjugating enzyme E2L 6	1.0	0.902	0.978
59	1.59E-10	3.2	DIAPH2	diaphanous homolog 2 (Drosophila)	1.1	0.329	0.003
59	1.50E-9	2.4	ALDOA	aldolase A, fructose bisphosphate	1.0	0.759	4.91E-005
59	1.69E-8	3.4	SLC15A4	solute carrier family 15, member 4	1.0	0.651	0.108

Table 2
Top 20 genes overexpressed at the mRNA level in the γ -HIF2 α -M3 TG+ vs. WT kidneys by RNAseq

The list of the top 20 genes overexpressed at the RNA level in the γ -HIF2 α -M3 TG+ vs. WT kidneys was compiled from the RNAseq results. The median fold change in mRNA levels of these genes in human ccRCC was compiled from Oncomine by combining five different sets of human ccRCC patient samples, totaling 175 patients (see Table 1). Genes that has no measurements from all five datasets were excluded from this list. One of these genes (Cd163l1*) shows overexpression (fold change>3) in the Oncomine datasets.

fold-change- γ -HIF2 α -M3/WT	t-test-BH-FDR-q-value	fisher-exact-R γ -HIF2 α -M3/WT-BH-FDR-q-value	Element ID	Gene Description	Human ccRCC/normal kidney Median Fold Change
121.37	0	0	ENSMUSG00000025515	mucin 2	-1.09
57.1	4.21E-006	4.46E-147	ENSMUSG00000034486	gastrulation brain homeobox 2	-1.02
48.32	0	0	ENSMUSG00000030111	alpha-2-macroglobulin	1.81
45.86	0	7.48E-083	ENSMUSG00000026468	LIM homeobox protein 4	1.77
36.31	2.03E-005	0	ENSMUSG00000050645	defensin beta 19	0.38
33.13	0	0	ENSMUSG00000032080	apolipoprotein A-IV	-1.23
21.74	0.01	0	ENSMUSG00000056296	synaptoporin	-1.15
19.73	0.01	1.71E-144	ENSMUSG00000024008	copine V; similar to Copine V	1.54
19.15	0.01	2.02E-042	ENSMUSG00000018893	myoglobin	-1.09
17.16	1.27E-005	0	ENSMUSG00000032081	apolipoprotein C-III	-1.7
17.14	0	0	ENSMUSG00000021708	RAS protein-specific guanine nucleotide-releasing factor 2	1.57
15.5	0.02	5.83E-090	ENSMUSG00000000731	autoimmune regulator (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy)	-1.08
14.24	0.03	1.05E-033	ENSMUSG00000042268	solute carrier family 26, member 9	-0.23
10.97	6.47E-005	9.41E-094	ENSMUSG00000025461	CD163 molecule-like 1 *	3.15
7.49	0.01	0	ENSMUSG00000038418	early growth response 1	-1.16
6.49	0.01	1.72E-038	ENSMUSG00000030236	solute carrier organic anion transporter family, member 1b2	-1.07
6.26	0.01	6.27E-010	ENSMUSG00000052468	peripheral myelin protein2	-1.14
6.14	0.01	2.65E-009	ENSMUSG00000054667	insulin receptor substrate 4; similar to insulin receptor substrate 4	-1.16
5.62	0.02	3.81E-011	ENSMUSG00000026959	glutamate receptor, ionotropic, NMDA1 (zeta 1)	1.34
5.39	0.05	1.46E-271	ENSMUSG00000024411	aquaporin 4	-1.01