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HIF-1 α and HIF-2 α have divergent roles in colon cancer

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Abstract

Hypoxia-inducible factor (HIF)-1 and HIF-2 are heterodimeric transcription factors that mediate the cellular response to hypoxia. Their key regulatory subunits, HIF-1 α and HIF-2 α , are induced similarly by hypoxia, but their functional roles in cancer may be distinct and isoform-specific. SW480 colon cancer cells with stable expression of siRNA to HIF-1 α or HIF-2 α or both were established. HIF-1 α -deficient cells displayed lower rates of proliferation and migration, but HIF-2 α -deficient cells exhibited enhanced anchorage independent growth in a soft agar assay. Xenograft studies revealed that HIF-1 α deficiency inhibited overall tumor growth, whereas deficiency of HIF-2 α stimulated tumor growth. In human colon cancer tissues, expression of HIF-1 α and to a lesser extent, HIF-2 α , was linked to upregulation of VEGF and tumor angiogenesis. However, loss of expression of HIF-2 α but not HIF-1 α was strongly correlated with advanced tumor stage. DNA microarray analysis identified distinct sets of HIF-1 α and HIF-2 α target genes that may explain these phenotypic differences. Collectively, these findings suggest that HIF isoforms may have differing cellular functions in colon cancer. In particular, HIF-1 α promoted the growth of SW480 colon cancer cells but HIF-2 α appeared to restrain growth. Consequently, therapeutic approaches that target HIF may need to consider these isoform-specific properties.

Keywords

colon cancer; HIF; angiogenesis

Hypoxia is an important environmental regulator of tumor angiogenesis and growth. Many of the adaptations to hypoxia are mediated by the activation of specific genes through hypoxia-inducible factor (HIF).^{1,2} The first HIF described (HIF-1) is a heterodimer of HIF-1 α and HIF-1 β .³ In normoxic conditions, HIF-1 α , a basic helix-loop-helix transcription factor, is hydroxylated at specific proline residues. This results in ubiquitination through an interaction with the von Hippel-Lindau tumor suppressor protein (pVHL) and proteasomal degradation.

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⁴ Under hypoxic conditions, proline hydroxylation is inhibited, preventing the association with pVHL. HIF-1 α accumulates and dimerizes with HIF-1 β , which then activates a specific set of genes by binding to hypoxia response elements in their promoter regions.

A structurally related protein, HIF-2 α , has been also described and partially characterized.⁵ HIF-2 α (also known as EPAS1) shares 48% overall amino acid identity with HIF-1 α and 83% identity in the basic helix-loop-helix domains. Like HIF-1 α , HIF-2 α accumulates in the presence of hypoxia, forms a heterodimer with HIF-1 β and binds to hypoxia response elements. Although they are similarly induced by hypoxia and recognize the same DNA sequence elements, several reports suggest that HIF-1 α and HIF-2 α have distinct expression patterns and functions.⁷ In particular, although HIF-1 α has broad activity, the response to HIF-2 α may be restricted to specific cell types.⁸ Expression of HIF-2 α was initially thought to be limited to vascular endothelial cells during embryonic development.⁵ However, it has since been reported that HIF-2 α is expressed in kidney fibroblasts, hepatocytes, intestinal epithelial cells, pancreatic interstitial cells, cardiac myocytes and lung type II pneumocytes.⁹ However, the relative importance of these genes can vary with the cell type; for example, the response to hypoxia is largely mediated by HIF-1 α in endothelial and breast cancer cells but by HIF-2 α in renal carcinoma cells.¹⁰ HIF-2 α over expression is important in the development of renal carcinoma in patients with the von Hippel Lindau syndrome; and in this setting, HIF-2 α may act as a renal cancer oncogene.¹¹ In neuroblastomas, expression of HIF-2 α is associated with more aggressive disease.¹² However, other studies in glioblastomas suggested that over expression of HIF-2 α enhances angiogenesis but reduces tumor growth.¹³ Thus, there is accumulating evidence that the importance of HIF-1 α and HIF-2 α in the response to hypoxia varies among tumor types and that their biological functions may differ. We were interested in the roles of HIF-1 α and HIF-2 α in colonic tumorigenesis. Although the role of HIF-1 α in colorectal cancer has been characterized,^{14–18} there is a limited understanding of the role of HIF-2 α .

Material and methods

Cell culture

SW480 and DLD-1 cells were obtained from American Type Culture Collection (Manassas, VA). SW480 cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin, and DLD1 cells were cultured in RPMI with 10% FBS and 1% penicillin-streptomycin (GIBCO, Carlsbad, CA). Hypoxic conditions were achieved by culturing cells in a sealed hypoxia chamber (Billups-Rothenberg, Del Mar, CA) after flushing with 1% O₂, 5% CO₂ and 94% N₂. To minimize the effect of serum growth factors, the cell culture medium was switched to serum-free Ultraculture (BioWhittaker) before the cells were subjected to hypoxia.

Plasmid construction and retroviral infection

Plasmids expressing HIF-1 α siRNA or control (designated pSR 1K or pSR ctrl) containing a puromycin resistance gene were generated as previously described.¹⁹ Plasmids expressing HIF-2 α siRNA were generated as below. Briefly, the pSUPER GFP neo vector (Oligoengine, Seattle, WA) that expresses siRNA under the control of the polymerase-III H1-RNA promoter was used after inserting DNA oligonucleotides between the *Bgl* II and *Hind* III restriction sites according to the manufacturer's protocol. Plasmids expressing HIF-2 α siRNA or control are named pSR GFP 2K or pSR GFP ctrl. To generate SW480 cells retrovirally transduced with HIF-1 α and/or HIF-2 α siRNA, either pSR 1K or pSR ctrl was first transfected into 293GPG packaging cells, and filtered conditioned media was added to SW480 cells using polybrene (Sigma, Milwaukee, WI). The same procedure was also performed using pSR GFP 2K or pSR GFP ctrl. After selection, with 2.0 μ g/ml of puromycin and 500 μ g/mg of G418, cells were immediately used for the experiments. The experimental cells containing either HIF-1 α siRNA,

HIF-2 α siRNA or both HIF-1 α siRNA and HIF-2 α siRNA are designated SW480 1K, SW480 2K and SW480 1K2K, respectively, and control cells that contain both pSR ctrl and pSR GFP ctrl are designated SW480 WT. The siRNA sequences for HIF-1 α ¹⁹ and HIF-2 α ²⁰ have been previously described.

***In vitro* proliferation and migration assays**

To determine the effect of HIF-1 α and HIF-2 α expression on cell proliferation, we cultured cells (5,000 per well) in a 24-well plate in standard growth media. Cells were counted (3 wells per time point) on days 3 and 5 after plating. To determine if HIF-1 α and HIF-2 α expression influences cell migration, we cultured SW480 WT, SW480 1K, SW480 2K and SW480 1K2K cells in BIOCOAT Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ), essentially as described.²¹ Cells were cultured under 1% O₂ for 24 hr, and 2.5×10^4 cells per well were plated and cell numbers were determined 16 hr later. All of the above experiments were repeated at least 3 times.

Soft agar colony assay

A soft agar assay was performed as previously reported.²² SW480 cells (6×10^4 cells per well) retrovirally infected with the indicated plasmids were mixed with culture medium containing 0.6% agar to result in a final agar concentration of 0.3%. 27 ml samples of this cell suspension were immediately plated onto a 100 mm dish coated with 0.6% agar in tissue culture medium (9 ml per dish) and cultured at 37°C with 5% CO₂. After 3 weeks, colonies larger than 100 μ m in diameter were counted. The studies were performed in triplicate.

Tumor growth in nude mice

Exponentially growing SW480 WT, SW480 1K, SW480 2K and SW480 1K2K cells were collected by trypsinization, and 1.0×10^7 cells were resuspended in high concentration Matrigel (BD Biosciences), diluted in serum-free medium to 50% final concentration and subcutaneously injected into 6-week-old CD1 female nude mice according to a protocol approved by the Massachusetts General Hospital Animal Care and Use Committee. Each mouse was injected at 2 sites, and 5 mice were studied in each experimental group. Tumor growth was monitored by weekly inspections. Animals were euthanized at 42 days after injection, tumors were removed and weighed and all major organs (liver, lung and spleen) were also inspected for the presence of tumor cells. Representative sections of each tumor were fixed in formalin and paraffin embedded for further analysis, and the remaining sections were frozen on dry ice for subsequent RNA extraction.

Western blot

We performed western blotting as previously described.²³ Briefly, 20 to 80 μ g of protein extracts were resolved on a 7% NuPAGE Tris-Acetate polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The blots were probed with a HIF-1 α antibody (BD transduction laboratories) at 1:500 or HIF-2 α antibody (Novus Biologicals, Littleton, CO) at 1:1,000. Immunoreactive proteins were visualized with the Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA).

Tumor samples

Formalin fixed, paraffin-embedded tissues from 63 consecutive patients with colon cancer were retrieved from the files of the Department of Pathology, Massachusetts General Hospital. The patients and tumor characteristics are shown in Table I. This protocol was approved by the Institutional Review Board of the Massachusetts General Hospital.

Immunohistochemistry

Paraffin sections of human colon cancers containing both normal mucosa and the invasive front of the tumor tissue and also SW480 xenografts were analyzed by immunohistochemical staining. Breast cancer tissue specimens from selected patients were used as a positive control for HIF-1 α and HIF-2 α immunohistochemistry, and squamous head and neck cancers were used as a positive control for CCNG2. Briefly, slides were deparaffinized, rehydrated in graded alcohol and placed in phosphate-buffered saline solution. Antigen retrieval was performed by autoclaving the sections in citrate buffer (pH 6.0, Dako) for HIF-1 α or in Nuclear Decloaker (pH 9.5, Biocare Medical, Concord, CA) for HIF-2 α and Cyclin G2. Endogenous peroxidase was blocked by incubating the slides in 0.3% H₂O₂ for 10 min. Sections were incubated for 30 min with normal serum from donor species of secondary antibody followed by an overnight incubation with the primary antibodies [anti-HIF1 α (clone H1alpha67) and anti-HIF2 α (clone ep190b); Novus Biologicals, Littleton, CO] at 1:200. Link antibody was applied for 30 min. Sections were incubated for 30 min with Avidin–Biotinylated enzyme Complex (VECTASTAIN[®] ABC kits, Vector lab, Burlingame, CA). Finally, peroxidase activity was detected with DAB solution (Vector DAB substrate kit, Vector) for 3 min. In between the steps, slides were washed 3 times in phosphate-buffered saline. Counterstaining was performed with Meyer's hematoxylin. A positive and a negative control slide were always included in each immunostaining. Tumor microvessel density was detected by CD31 immunohistochemistry and calculated as previously described.²⁴ Mouse monoclonal antibody against PECAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:200, for 14 hr at 4°C), rabbit polyclonal antibody against VEGF (Santa Cruz Biotechnology; dilution 1:100, for 14 hr at 4°C), and antibody against cyclin G2 (Santa Cruz Biotechnology, dilution 1:200, for 14 hr at 4°C) were used. For VEGF immunohistochemistry, colon cancer tissues that were known to over express VEGF were used as a positive control. Two investigators who were blinded to the patient's clinical information scored all slides independently, and the differences between the 2 observers were resolved at conference microscopy. For HIF-1 α and HIF-2 α , nuclear staining was graded from 0 to 2+, with 0 representing no detectable nuclear expression (negative control) and 2+ representing the strongest nuclear stain equivalent to that of a positive control. Cytoplasmic expression of VEGF was graded from 0 to 3+, with 0 representing no detectable stain (negative control) and 3+ representing the strongest stain (positive control). In tumors where there was heterogeneous staining, the percentage of areas exhibiting each grade was determined, and then an average final score was calculated.

cDNA microarray analysis

SW480 WT, SW480 1K and SW480 2K cells were used for microarray experiments. Cells were incubated in 1% O₂ for 16 hr, and mRNA was collected. SW480 WT cells grown in normoxic conditions were used as control. We performed sample preparation and processing procedures as described previously.²⁵ We hybridized the labeled samples to the complete Affymetrix human U133 Plus 2.0 GeneChip set (HG-U133A 2.0). Analysis was performed using R version 2.4.0 and bioconductor modules version 1.9.²⁶ Microarray normalization was performed using GCRMA module²⁷ and present/absent calls were calculated using Affymetrix MAS5 package. Only genes with at least one present call and at least one expression value above 100 were retained. Genes that showed more than 2-fold upregulation from SW480 WT in normoxia to SW480 WT in hypoxia and had an expression value above 100 in hypoxia were defined as “hypoxia induced transcripts.” These transcripts were partitioned into 4 groups depending on their response to the HIF-1 α and HIF-2 α siRNAs, relative to their expression levels in hypoxia. Genes with >30% decrease in expression with HIF-1 α siRNA but not with HIF-2 α siRNA were classified as HIF-1 dependent and vice versa for HIF-2 dependent genes. Genes with >30% decrease in expression with both HIF-1 α and HIF-2 α siRNAs were classified as HIF-1 and HIF-2 dependent, and the remaining were HIF independent. Because some genes are represented by multiple probes on the microarray, the classification was corrected by using

all probes matching the same gene. Heatmaps were generated for each group separately using hierarchical clustering.

Quantitative PCR

Quantitative PCR was performed as previously described.²⁵ Primer sequences for ANGPTL4 and CCNG2 are available upon request.

Transient transfection

Transient transfection of a HIF-2 α (EPAS1) vector (gift of Richard Bruick) was performed with Lipofectamine 2000, as previously described.²⁵

Statistical analysis

We performed statistical analyses with a 2-tailed, unpaired Student's *t*-test for the *in vitro* and *in vivo* studies. We used both the Student's *t*-test and Fisher's exact probability test for the immunohistochemical study.

Results

Roles of HIF-1 α and HIF-2 α on cellular proliferation and migration *in vitro*

In most cancer cell lines, hypoxia induces the expression of primarily HIF-1 α or HIF-2 α . The SW480 colon cancer cell line was selected because HIF-1 α and HIF-2 α were both strongly induced by hypoxia (Fig. 1). In several other colon cell lines tested (HT29, DLD1, Caco2), induction of HIF-2 α by hypoxia was absent or minimal.^{19,25} We analyzed the roles of these 2 isoforms *in vitro* using SW480 cells in which HIF-1 α (SW480 1K) or HIF-2 α (SW480 2K) was stably knocked down (Fig. 1). To determine proliferative properties, 5,000 cells each were seeded in 24-well dishes and cultured in normoxic conditions. After 5 days, the cell count was $52,750 \pm 1,871$ for SW480 WT control cells. In contrast, the SW480 1K cell count was significantly decreased ($30,500 \pm 3,240$, $p < 0.01$). There was no change in the number of SW480 2K cells ($54,000 \pm 4,320$) (Fig. 2a). When both HIF-1 and HIF-2 were knocked down simultaneously, an intermediate phenotype was observed (cell count = $42,000 \pm 5,888$). Thus, HIF-1 α appears to provide a proliferative stimulus, even in the absence of hypoxia.

Soft agar assays were then performed to determine whether these effects were also observed in a 3-dimensional anchorage-independent culture system. In soft agar assays, the number of colonies of SW480 WT control cells was 26.5 ± 3.5 per high power field (HPF). When HIF-1 α was knocked down, there was no significant change in colony number (24.8 ± 6.2 per HPF). However, in the absence of HIF-2 α , the colony number doubled to 53.2 ± 4.6 per HPF (Fig. 2b). This was a statistically significant increase in the number of colonies of SW480 2K cells compared to SW480 WT control cells ($p < 0.01$), suggesting that HIF-2 α normally suppresses anchorage-independent growth in SW480 cells. When both HIF-1 α and HIF-2 α were knocked down, an intermediate phenotype was observed (43.3 ± 5.3 colonies, $p = 0.02$).

The influence of HIF-1 α and HIF-2 α expression on cell migration was then assessed using a modified Boyden chamber assay. 2.5×10^4 cells incubated in hypoxia were seeded onto the chamber. 203.3 ± 18.2 SW480 WT cells per HPF migrated through an 8 μ m pore after 16 hr. There was a dramatic decrease in the number of SW480 1K cells that migrated through the pore (27.6 ± 3.5 cells per HPF, $p < 0.01$). In contrast, the migratory capabilities of the SW480 2K cells were unchanged (288.7 ± 14.0 cells per HPF) (Fig. 2c). In SW480 1K2K cells, migration was significantly impaired (60.3 ± 13.0 cells per HPF, $p < 0.01$), and the phenotype resembled the behavior of the SW480 1K cells. The consistent and significant decrease in the motility of cells with reduced HIF-1 α expression ($p < 0.01$) suggested that HIF-1 α is also a positive regulator of cellular migration.

Effects of HIF-1 α and HIF-2 α on tumorigenicity in nude mice

To determine if HIF-1 α and HIF-2 α expression correlated with tumor growth and invasion *in vivo*, 1×10^7 SW480 WT, SW480 1K or SW480 2K cells were subcutaneously injected into the flanks of female nude mice, and tumor growth and metastasis were assessed. In addition, cells deficient in both HIF-1 α and HIF-2 α (SW480 1K2K) were analyzed to define the interplay between the 2 isoforms *in vivo*. The mean tumor weight at 6 weeks after injection of the SW480 WT control cells was 312.9 ± 49.0 mg. Cells deficient in HIF-1 α formed significantly smaller tumors at 6 weeks (134.6 ± 27.8 mg, $p < 0.01$). However, the SW480 cells that were deficient in HIF-2 α formed significantly larger tumors (580.8 ± 84.7 mg, $p < 0.01$) (Fig. 3a). The mean tumor volumes over 6 weeks are shown in Figure 3b, and representative tumors and H & E stains are illustrated in Figure 3c. The tumors were smaller in the absence of HIF-1 α , which is consistent with previous reports,^{25,28} but tumors were significantly larger in the absence of HIF-2 α . These findings are consistent with the *in vitro* results. Interestingly, although silencing of HIF-2 α appeared to enhance tumorigenesis, this effect was overridden when HIF-1 α was simultaneously knocked-down (Figs. 3a and 3b). The size of the SW480 1K2K tumors was similar to SW480 1K tumors. No distant metastases were observed in any of the experimental groups. Immunohistochemistry performed for HIF-1 α and HIF-2 α verified the knock-down effects in the tumor xenografts (Supp. Info. Fig. 1).

Vascular endothelial growth factor (VEGF) can be induced by both HIF-1 and HIF-2, and the expression of VEGF in the tumor xenografts was measured by ELISA. The VEGF protein level was 923.9 ± 64.1 pg/mg total protein in the control SW480 WT tumors. The levels were 49% lower in the SW480 1K xenografts (473.6 ± 52.0 pg/mg total protein, $p < 0.01$) and 20% lower in SW480 2K xenografts (740.2 ± 132.5 pg/mg total protein, $p < 0.01$). The VEGF level in SW480 1K2K tumors was 476.8 ± 163.3 pg/mg total protein, similar to the SW480 1K tumors (Fig. 3d). Reduced expression of either HIF-1 α or HIF-2 α was linked to reduced protein levels of VEGF ($p < 0.01$ compared with SW480 WT tumors), although HIF-1 α appeared to have a greater impact. The microvessel density (MVD) in SW480 WT, SW480 1K, SW480 2K and SW480 1K2K tumors was then determined. CD31 immunohistochemistry revealed a baseline MVD in control SW480 WT xenografts of 35.1 ± 9.7 vessels per HPF. In both SW480 1K and SW480 2K xenografts, the MVD decreased (24.1 ± 8.1 vessels per HPF and 23.0 ± 10.6 vessels per HPF, respectively, $p < 0.05$), and the MVD was similar in the double knockdown (20.9 ± 5.9 vessels per HPF, $p < 0.05$). Loss of expression of either HIF-1 α or HIF-2 α was therefore associated with reduced vascularization in SW480 xenografts (Fig. 3e).

Finally, PCNA immunostaining was performed to assess alterations in cellular proliferation. The percentage of PCNA positive cells in the control SW480 WT xenografts was $58.8 \pm 10.8\%$. There was no significant difference in the SW480 1K tumors ($49.6 \pm 6.7\%$). However, the SW480 2K tumors exhibited significantly higher rates of PCNA positivity ($86.3 \pm 5.0\%$, $p < 0.01$). Again, the PCNA levels in the SW480 1K2K tumors ($63.8 \pm 8.5\%$) mirrored that of the SW480 1K tumors. Thus, cellular proliferation was significantly increased in HIF-2 α knockdown tumors, suggesting that the increase in size of HIF-2 α deficient tumors was at least partially a consequence of increased cellular proliferation rates (Fig. 3f).

HIF-1 α and HIF-2 α expression in primary human colon carcinomas

To clarify the roles of the HIF-1 α and HIF-2 α isoforms in human colon cancer, we performed HIF-1 α , HIF-2 α , VEGF and CD31 immunohistochemistry on 63 colon cancer specimens. Representative staining results are depicted in Figure 4. HIF-1 α and HIF-2 α expression were graded on a 0–2+ scale. Normal mucosa shows weak staining for both HIF-1 α and HIF-2 α , and their expression levels were scored as 1+ as an internal control. Tumor samples showing more than 1+ staining were considered to have strong staining. For HIF-1 α , 36 (57.1%) tumors exhibited strong staining (>1+) and 27 (42.9%) exhibited weak ($\leq 1+$) staining. For HIF-2 α , 28

(44.4%) tumors exhibited strong ($>1+$) and 35 (55.6%) exhibited weak ($\leq 1+$) expression. In 37 (58.7%) patients, expression of both HIF-1 α and HIF-2 α was concordant (both strong or both weak). However, in 26 patients (41.3%) the expression patterns were divergent (HIF-1 α strong and HIF-2 α weak *or* HIF-1 α weak and HIF-2 α strong), suggesting that HIF-1 α and HIF-2 α are frequently regulated in a differential manner.

To determine if there was an association between HIF-1 α or HIF-2 α expression and angiogenesis in human colon tumors, we compared HIF expression levels with VEGF and MVD levels (Tables II–V). We found a statistically significant correlation between HIF-1 α expression and VEGF ($p < 0.001$) as well as between HIF-1 α and microvessel density ($p = 0.001$). A similar trend was also found between HIF-2 α and VEGF as well as between HIF-2 α and MVD, although these did not reach statistical significance ($p = 0.08$ for HIF-2 α and VEGF, $p = 0.18$ for HIF-2 α and MVD). We then wanted to determine if there was an association between disease stage and HIF-1 α or HIF-2 α expression (Tables VI, VII). 54.3% of Stage 1 and 2 tumors exhibited strong HIF-1 α staining, and 60.7% of Stage 3 and 4 tumors exhibited strong HIF-1 α staining ($p = 0.40$). In contrast, 60.0% of Stage 1 and 2 tumors displayed strong HIF-2 α staining but only 25.0% of Stage 3 and 4 tumors exhibited strong HIF-2 α staining ($p = 0.004$). There was a significant inverse correlation between tumor stage and HIF-2 α expression whereas HIF-1 α expression was not associated with tumor stage. Finally, we examined the correlation between tumor stage and combined expression of HIF-1 α and HIF-2 α (Table VIII). Interestingly, advanced tumors were more likely to display strong HIF-1 α staining and weak HIF-2 α staining (70.6% of these cases were Stage 3 or 4), whereas earlier stage tumors were more likely to display weak HIF-1 α staining and strong HIF-2 α staining (77.8% of these cases were Stage 1 or 2, $p = 0.03$). This suggests that HIF-1 α and HIF-2 α do function in opposing manners in human colon cancer.

cDNA microarray studies reveal unique HIF-1 α and HIF-2 α targets

cDNA microarray studies were performed to characterize genes that may be differentially regulated by HIF-1 α and HIF-2 α in colon cancer. Total RNA was harvested from SW480 WT cells grown in normoxic and hypoxic conditions, and from SW480 1K and SW480 2K cells grown in hypoxia. Only genes with at least one present call and at least one expression value above 100 were retained, and this process reduced the number in the dataset from 54,675 probes to 24,501 probes. Genes that showed more than 2-fold upregulation from normoxia to hypoxia in SW480 WT cells and had an expression value above 100 in hypoxia were considered to be “hypoxia inducible transcripts,” and 1,030 transcripts were identified matching 830 unique gene symbols. These hypoxia inducible transcripts were divided into 4 different groups depending upon the response to siRNA to HIF-1 α or HIF-2 α or both. Several genes, represented by multiple probes, were present in more than 1 group and excluded from the analysis, resulting in 830 unique hypoxia inducible genes (HIF-1 α dependent = 177, HIF-2 α dependent = 72, HIF independent = 202, HIF-1 α and HIF-2 α dependent = 379; Figure 5, Supp. Info. Table I).

We were particularly interested in genes that may be selectively regulated by HIF-2 α because of the unique properties observed in HIF-2 α knockdown cells. Genes that have been previously described as targets of HIF-2 were identified in this group and included EGLN3²⁰ and CXCR4.²⁹ In addition, several known HIF-1 targets were also identified (PDK1,^{30,31} PGK1,³² and PFKFB3³³). We focused on 2 novel HIF-2 gene targets: angiopoietin-like 4 (ANGPTL4) and cyclin G2 (CCNG2). Quantitative-PCR was performed to validate these results of the microarray study. Figure 6 illustrates that in SW480 cells, both were induced by hypoxia (17.3 fold increase of ANGPTL4 mRNA and 5.73 fold increase of CCNG2 mRNA), and their expression was repressed to a greater degree by HIF-2 α siRNA (84.2% reduction for ANGPTL4 and 70.3% reduction for CCNG2) than by HIF-1 α siRNA (14.7% reduction for ANGPTL4 and 38.4% reduction for CCNG2). Immunohistochemistry for CCNG2 was

performed in the tumor xenografts, and loss of nuclear staining for CCNG2 was confirmed only in the SW480 2K xenografts (Supp. Info. Fig. 1). When HIF-2 α was transiently over expressed in an independent colon cancer cell line (DLD1), there was an 8.4 fold increase in ANGPTL4 mRNA levels and 2.0 fold increase in CCNG2 mRNA levels, as measured by qPCR. Both ANGPTL4 and CCNG2 have growth inhibitory properties,^{34–36} suggesting that ANGPTL4 and CCNG2 may potentially mediate some of the tumor suppressive effects of HIF-2 α in colon cancer cells.

Discussion

We have delineated distinct roles for the HIF-1 α and HIF-2 α isoforms in SW480 colon cancer cells. It has been suggested that the roles of HIF-1 α and HIF-2 α vary depending upon the tissue type. Although a link between HIF-1 α , VEGF, and microvessel density has been recognized in colon cancer,^{37,38} the contrasting role of HIF-2 α in this tumor type has not been previously characterized. The immunohistochemical expression patterns of HIF-1 α and HIF-2 α are not concordant in over 40% of human colon cancer cases, and the growth patterns of SW480 colon cancer cells *in vitro* and *in vivo* are sharply divergent based upon whether HIF-1 α or HIF-2 α is present.

To dissect the roles of these 2 isoforms, we examined SW480 colon cancer cells in which both isoforms are strongly induced by hypoxia. In this manner, their individual as well as combined roles could be analyzed. The selective knock-down of HIF-1 α resulted in lower rates of proliferation and migration *in vitro*, and when grown *in vivo* as xenografts, these cells exhibited a 57% reduction in tumor size. In contrast, the selective knock-down of HIF-2 α had no effect on cellular proliferation *in vitro*, but colony formation doubled in soft agar assays. In xenograft studies, cells deficient in HIF-2 α formed tumors that were nearly double in size compared to wild type cells. These effects on growth did not appear to be a consequence of alterations in tumor angiogenesis. Specifically, both HIFs regulated the levels of VEGF as well as microvessel density in a similar manner. In comparison to a previous study in which HIF-1 α was selectively knocked-down in DLD-1 cells,²⁵ the reduction in VEGF and MVD levels was greater in SW480 cells. Both cell lines carry mutant K-ras alleles,³⁹ but they differ in p53 status.^{39,40} p53 is a negative regulator of VEGF⁴¹ and HIF-1 α ,³⁸ and the functional p53 allele in DLD-1 cells may potentially explain this difference. Nevertheless, these findings imply that the sharp contrast in the HIF-1 α -deficient and HIF-2 α -deficient tumor phenotypes may reflect differential regulation of intrinsic cell autonomous properties.

These opposing roles of HIF-1 α and HIF-2 α are supported by studies of HIF expression in human colon cancer samples. HIF-1 α expression was relatively uniform among all tumor stages, but there was a significant loss of HIF-2 α staining in more advanced tumors. This is consistent with a “tumor suppressive” role for HIF-2 α in colon cancer. There has been one previous report of HIF-1 α and HIF-2 α immunostaining in colon tumors, and in contrast, a positive association was observed between HIF-2 α staining and advanced tumor stage.⁴² Although identical protocols and antibodies were utilized for immunohistochemical staining, the functional correlates in the this report support the hypothesis that HIF-2 α functions in a tumor suppressive capacity.

In other tumor types, there are examples in which HIF-1 α behaves as an oncogene whereas HIF-2 α behaves as a tumor suppressor. Over expression of HIF-2 α in glioma cells suppressed tumor growth, and HIF-2 α -deficient ES cells displayed enhanced growth as teratomas.¹³ In contrast, loss of expression of HIF-1 α frequently resulted in impaired growth in multiple tumor types.^{43–46} In renal cancer, however, HIF-1 α can behave in a distinct manner. Silencing of HIF-2 α with siRNA reduced the growth of the tumor,^{11,47} and over expression of HIF-2 α but

not HIF-1 α enhanced renal tumor growth,⁴⁸ suggesting that in some circumstances, HIF-2 α can function in an oncogenic capacity.

We were curious to better understand the cellular phenotypes when both isoforms were present or not. As the two had divergent functions, we sought to define whether one isoform was more dominant when both were expressed. In our system, we addressed this by knocking down both HIF-1 α and HIF-2 α simultaneously. These studies suggested that the HIF-1 α isoform had a more dominant phenotype *in vivo*, as the growth pattern of the double knockdown was similar to the HIF-1 α -knockdown as a xenograft, and an intermediate phenotype was not observed.

Understanding the differential activity of the HIF isoforms is an important issue in the biology of HIF. Both HIF-1 and HIF-2 are thought to recognize the same hypoxia response element in the promoters of hypoxia responsive genes, but the target genes activated by these 2 isoforms appear to differ. Several groups have characterized these differences through genome-wide analyses of HIF-1 α and HIF-2 α dependent genes with cDNA microarray.^{7,49,50} For example, Wang *et al.*⁷ asked whether 293 cells over expressing HIF-1 α exhibited differing gene expression patterns than those over expressing HIF-2 α . They identified 21 genes that were influenced by both HIF-1 α and HIF-2 α , 14 that were preferentially upregulated by HIF-1 α , and 10 that were upregulated by HIF-2 α . However, they did not consider the effects of hypoxia and an artificial over expression system was utilized in the study.

In this study, we addressed this question using colon cancer cells in which either HIF-1 α , HIF-2 α , or both were stably knocked-down. We were particularly interested in the group of HIF-2 α dependent genes because of HIF-2 α 's unique effects on colonic tumorigenesis. Among the hypoxia inducible genes found to be regulated by HIF-2 α , we focused on cyclin G2 (CCNG2) and angiopoietin-like 4 (ANGPTL4). Quantitative RT-PCR confirmed both genes to be hypoxia-inducible and HIF-2 α dependent. CCNG2 is unique member of the cyclin family, and is reported to have cell cycle inhibitory functions.^{51,52} Cyclin G2 is downregulated in oral cancer, and forced expression of cyclin G2 inhibited cellular proliferation and colony formation of SCC15 squamous cell carcinoma cells.³⁶ ANGPTL4 is a secreted protein of the angiopoietin-like family and known to be hypoxia inducible.⁵³ Recently, ANGPTL4 has been shown to have inhibitory effects on tumor cell migration and tumor metastasis.^{34,35} These molecules represent novel HIF-2 α targets that may partially contribute to its tumor suppressive function in colon cancer.

Currently, the precise transcriptional mechanisms underlying the differential activity of HIF-1 α and HIF-2 α are still unknown. Aprelikova *et al.*⁵⁰ suggested that structural differences in the transcription factor could account for the differential regulation by HIF-1 α and HIF-2 α in MCF7 breast cancer cells. For example, the ETS transcription factor family was found to interact with HIF-2 α . More recently, differential interactions between the 2 HIF isoforms and c-Myc have been reported as another explanation for their divergent cellular functions.^{54,55} Others have suggested that the differences between HIF-1 α and HIF-2 α driven transcription may be dependent upon the cellular microenvironment, such as duration of exposure to hypoxia or the specific concentration of oxygen.¹²

In summary, our studies have demonstrated divergent cellular functions for the HIF-1 α and HIF-2 α isoforms in colon cancer. This has important implications for the implementation of HIF inhibitors as a therapeutic approach in cancer. Targeting HIF has been proposed as an attractive strategy to block tumor angiogenesis and simultaneously disable additional mechanisms for tumor survival in a hypoxic microenvironment. Based on this study, an inhibitor that nonselectively targeted both HIF-1 α and HIF-2 α would be predicted to have a net therapeutic benefit. However, some caution may be warranted if HIF-2 α was more selectively targeted as this could potentially enhance colon tumor growth. Approaches to block

HIF are entering into clinical trials,^{56,57} and it will be critical to define their selectivity for HIF isoforms.

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References

1. Semenza GL. HIF-1 and human disease: one highly involved factor. *Genes Dev* 2000;14:1983–91. [PubMed: 10950862]
2. Ratcliffe PJ, Pugh CW, Maxwell PH. Targeting tumors through the HIF system. *Nat Med* 2000;6:1315–16. [PubMed: 11100107]
3. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* 1992;12:5447–54. [PubMed: 1448077]
4. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 1999;399:271–5. [PubMed: 10353251]
5. Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev* 1997;11:72–82. [PubMed: 9000051]
6. Ema M, Taya S, Yokotani N, Sogawa K, Matsuda Y, Fujii-Kuriyama Y. A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1 α regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc Natl Acad Sci USA* 1997;94:4273–8. [PubMed: 9113979]
7. Wang V, Davis DA, Haque M, Huang LE, Yarchoan R. Differential gene up-regulation by hypoxia-inducible factor-1 α and hypoxia-inducible factor-2 α in HEK293T cells. *Cancer Res* 2005;65:3299–306. [PubMed: 15833863]
8. Akeno N, Czyzyk-Krzeska MF, Gross TS, Clemens TL. Hypoxia induces vascular endothelial growth factor gene transcription in human osteoblast-like cells through the hypoxia-inducible factor-2 α . *Endocrinology* 2001;142:959–62. [PubMed: 11159870]
9. Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, et al. Widespread hypoxia-inducible expression of HIF-2 α in distinct cell populations of different organs. *Faseb J* 2003;17:271–3. [PubMed: 12490539]
10. Sowter HM, Raval RR, Moore JW, Ratcliffe PJ, Harris AL. Predominant role of hypoxia-inducible transcription factor (Hif)-1 α versus Hif-2 α in regulation of the transcriptional response to hypoxia. *Cancer Res* 2003;63:6130–4. [PubMed: 14559790]
11. Kondo K, Kim WY, Lechpammer M, Kaelin WG Jr. Inhibition of HIF2 α is sufficient to suppress pVHL-defective tumor growth. *PLoS Biol* 2003;1:E83. [PubMed: 14691554]
12. Holmquist-Mengelbier L, Fredlund E, Lofstedt T, Noguera R, Navarro S, Nilsson H, Pietras A, Vallon-Christersson J, Borg A, Gradin K, Poellinger L, Pahlman S. Recruitment of HIF-1 α and HIF-2 α to common target genes is differentially regulated in neuroblastoma: HIF-2 α promotes an aggressive phenotype. *Cancer Cell* 2006;10:413–23. [PubMed: 17097563]
13. Acker T, Diez-Juan A, Aragones J, Tjwa M, Brusselmans K, Moons L, Fukumura D, Moreno-Murciano MP, Herbert JM, Burger A, Riedel J, Elvert G, et al. Genetic evidence for a tumor suppressor role of HIF-2 α . *Cancer Cell* 2005;8:131–41. [PubMed: 16098466]
14. Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G, Iyer N, LaRusch J, Pak B, Taghavi P, Semenza GL. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res* 2003;63:1138–43. [PubMed: 12615733]
15. Jubb AM, Pham TQ, Hanby AM, Frantz GD, Peale FV, Wu TD, Koeppen HW, Hillan KJ. Expression of vascular endothelial growth factor, hypoxia inducible factor 1 α , and carbonic anhydrase IX in human tumours. *J Clin Pathol* 2004;57:504–12. [PubMed: 15113858]

16. Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. *J Biol Chem* 2002;277:38205–11. [PubMed: 12149254]
17. Dang DT, Chen F, Gardner LB, Cummins JM, Rago C, Bunz F, Kantsevov SV, Dang LH. Hypoxia-inducible factor-1 α promotes non-hypoxia-mediated proliferation in colon cancer cells and xenografts. *Cancer Res* 2006;66:1684–936. [PubMed: 16452228]
18. Giles RH, Lolkema MP, Snijckers CM, Belderbos M, van der Groep P, Mans DA, van Beest M, van Noort M, Goldschmeding R, van Diest PJ, Clevers H, Voest EE. Interplay between VHL/HIF1 α and Wnt/ β -catenin pathways during colorectal tumorigenesis. *Oncogene* 2006;25:3065–70. [PubMed: 16407833]
19. Mizukami Y, Li J, Zhang X, Zimmer MA, Iliopoulos O, Chung DC. Hypoxia-inducible factor-1-independent regulation of vascular endothelial growth factor by hypoxia in colon cancer. *Cancer Res* 2004;64:1765–72. [PubMed: 14996738]
20. Aprelikova O, Chandramouli GV, Wood M, Vasselli JR, Riss J, Maranchie JK, Linehan WM, Barrett JC. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. *J Cell Biochem* 2004;92:491–501. [PubMed: 15156561]
21. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410:50–6. [PubMed: 11242036]
22. Krop I, Parker MT, Bloushtain-Qimron N, Porter D, Gelman R, Sasaki H, Maurer M, Terry MB, Parsons R, Polyak K. HIN-1, an inhibitor of cell growth, invasion, and AKT activation. *Cancer Res* 2005;65:9659–69. [PubMed: 16266985]
23. Imamura T, Kanai F, Kawakami T, Amarsanaa J, Ijichi H, Hoshida Y, Tanaka Y, Ikenoue T, Tateishi K, Kawabe T, Arakawa Y, Miyagishi M, et al. Proteomic analysis of the TGF- β signaling pathway in pancreatic carcinoma cells using stable RNA interference to silence Smad4 expression. *Biochem Biophys Res Commun* 2004;318:289–96. [PubMed: 15110786]
24. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 1991;324:1–8. [PubMed: 1701519]
25. Mizukami Y, Jo WS, Duerr EM, Gala M, Li J, Zhang X, Zimmer MA, Iliopoulos O, Zukerberg LR, Kohgo Y, Lynch MP, Rueda BR, Chung DC. Induction of interleukin-8 preserves the angiogenic response in HIF-1 α -deficient colon cancer cells. *Nat Med* 2005;11:992–7. [PubMed: 16127434]
26. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004;5:R80. [PubMed: 15461798]
27. Wu Z, Irizarry RA, Gentleman R, Martinez-Murillo F, Spencer F. A model based background adjustment for oligonucleotide expression arrays. *J Am Stat Assoc* 2004;99:909–917.
28. Chang Q, Qin R, Huang T, Gao J, Feng Y. Effect of antisense hypoxia-inducible factor 1 α on progression, metastasis, and chemosensitivity of pancreatic cancer. *Pancreas* 2006;32:297–305. [PubMed: 16628086]
29. Liu YL, Yu JM, Song XR, Wang XW, Xing LG, Gao BB. Regulation of the chemokine receptor CXCR4 and metastasis by hypoxia-inducible factor in non small cell lung cancer cell lines. *Cancer Biol Ther* 2006;5:1320–6. [PubMed: 16929169]
30. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* 2006;3:177–85. [PubMed: 16517405]
31. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab* 2006;3:187–97. [PubMed: 16517406]
32. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* 1994;269:23757–63. [PubMed: 8089148]
33. Minchenko A, Leshchinsky I, Opentanova I, Sang N, Srinivas V, Armstead V, Caro J. Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-

- bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect. *J Biol Chem* 2002;277:6183–7. [PubMed: 11744734]
34. Cazes A, Galaup A, Chomel C, Bignon M, Brechot N, Le Jan S, Weber H, Corvol P, Muller L, Germain S, Monnot C. Extracellular matrix-bound angiopoietin-like 4 inhibits endothelial cell adhesion, migration, and sprouting and alters actin cytoskeleton. *Circ Res* 2006;99:1207–15. [PubMed: 17068295]
 35. Galaup A, Cazes A, Le Jan S, Philippe J, Connault E, Le Coz E, Mekid H, Mir LM, Opolon P, Corvol P, Monnot C, Germain S. Angiopoietin-like 4 prevents metastasis through inhibition of vascular permeability and tumor cell motility and invasiveness. *Proc Natl Acad Sci USA* 2006;103:18721–6. [PubMed: 17130448]
 36. Kim Y, Shintani S, Kohno Y, Zhang R, Wong DT. Cyclin G2 dysregulation in human oral cancer. *Cancer Res* 2004;64:8980–6. [PubMed: 15604262]
 37. Kuwai T, Kitadai Y, Tanaka S, Onogawa S, Matsutani N, Kaio E, Ito M, Chayama K. Expression of hypoxia-inducible factor-1alpha is associated with tumor vascularization in human colorectal carcinoma. *Int J Cancer* 2003;105:176–81. [PubMed: 12673675]
 38. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, Bedi A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev* 2000;14:34–44. [PubMed: 10640274]
 39. Gayet J, Zhou XP, Duval A, Rolland S, Hoang JM, Cottu P, Hamelin R. Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. *Oncogene* 2001;20:5025–32. [PubMed: 11526487]
 40. Rochette PJ, Bastien N, Lavoie J, Guerin SL, Drouin R. SW480, a p53 double-mutant cell line retains proficiency for some p53 functions. *J Mol Biol* 2005;352:44–57. [PubMed: 16061257]
 41. Mukhopadhyay D, Tsiokas L, Sukhatme VP. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. *Cancer Res* 1995;55:6161–5. [PubMed: 8521408]
 42. Yoshimura H, Dhar DK, Kohno H, Kubota H, Fujii T, Ueda S, Kinugasa S, Tachibana M, Nagasue N. Prognostic impact of hypoxia-inducible factors 1alpha and 2alpha in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. *Clin Cancer Res* 2004;10:8554–60. [PubMed: 15623639]
 43. Chen J, Zhao S, Nakada K, Kuge Y, Tamaki N, Okada F, Wang J, Shindo M, Higashino F, Takeda K, Asaka M, Katoh H, et al. Dominant-negative hypoxia-inducible factor-1 alpha reduces tumorigenicity of pancreatic cancer cells through the suppression of glucose metabolism. *Am J Pathol* 2003;162:1283–91. [PubMed: 12651620]
 44. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev* 1998;12:149–62. [PubMed: 9436976]
 45. Kung AL, Zabludoff SD, France DS, Freedman SJ, Tanner EA, Vieira A, Cornell-Kennon S, Lee J, Wang B, Wang J, Memmert K, Naegeli HU, et al. Small molecule blockade of transcriptional coactivation of the hypoxia-inducible factor pathway. *Cancer Cell* 2004;6:33–43. [PubMed: 15261140]
 46. Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature* 1997;386:403–7. [PubMed: 9121557]
 47. Zimmer M, Doucette D, Siddiqui N, Iliopoulos O. Inhibition of hypoxia-inducible factor is sufficient for growth suppression of VHL^{-/-} tumors. *Mol Cancer Res* 2004;2:89–95. [PubMed: 14985465]
 48. Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Mol Cell Biol* 2005;25:5675–86. [PubMed: 15964822]
 49. Elvidge GP, Glenny L, Appelhoff RJ, Ratcliffe PJ, Ragoussis J, Gleadow JM. Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1alpha, HIF-2alpha, and other pathways. *J Biol Chem* 2006;281:15215–26. [PubMed: 16565084]

50. Aprelikova O, Wood M, Tackett S, Chandramouli GV, Barrett JC. Role of ETS transcription factors in the hypoxia-inducible factor-2 target gene selection. *Cancer Res* 2006;66:5641–7. [PubMed: 16740701]
51. Arachchige Don AS, Dallapiazza RF, Bennin DA, Brake T, Cowan CE, Horne MC. Cyclin G2 is a centrosome-associated nucleocytoplasmic shuttling protein that influences microtubule stability and induces a p53-dependent cell cycle arrest. *Exp Cell Res* 2006;312:4181–204. [PubMed: 17123511]
52. Bennin DA, Don AS, Brake T, McKenzie JL, Rosenbaum H, Ortiz L, DePaoli-Roach AA, Horne MC. Cyclin G2 associates with protein phosphatase 2A catalytic and regulatory B' subunits in active complexes and induces nuclear aberrations and a G1/S phase cell cycle arrest. *J Biol Chem* 2002;277:27449–67. [PubMed: 11956189]
53. Lal A, Peters H, St Croix B, Haroon ZA, Dewhirst MW, Strausberg RL, Kaanders JH, van der Kogel AJ, Riggins GJ. Transcriptional response to hypoxia in human tumors. *J Natl Cancer Inst* 2001;93:1337–43. [PubMed: 11535709]
54. Gordan JD, Bertout JA, Hu CJ, Diehl JA, Simon MC. HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. *Cancer Cell* 2007;11:335–47. [PubMed: 17418410]
55. Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, Huang LE. HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *Embo J* 2004;23:1949–56. [PubMed: 15071503]
56. Creighton-Gutteridge M, Cardellina JH II, Stephen AG, Rapisarda A, Uranchimeg B, Hite K, Denny WA, Shoemaker RH, Melillo G. Cell type-specific, topoisomerase II-dependent inhibition of hypoxia-inducible factor-1alpha protein accumulation by NSC 644221. *Clin Cancer Res* 2007;13:1010–18. [PubMed: 17289897]
57. Shin DH, Kim JH, Jung YJ, Kim KE, Jeong JM, Chun YS, Park JW. Preclinical evaluation of YC-1, a HIF inhibitor, for the prevention of tumor spreading. *Cancer Lett* 2007;255:107–16. [PubMed: 17502124]

Figure 1.

Establishment of SW480 colon cancer cells stably expressing HIF-1 α siRNA and HIF-2 α siRNA. Plasmids expressing either HIF-1 α or HIF-2 α siRNA were introduced retrovirally, and western blots were performed after exposure to hypoxia for 8 hr. 20 μ g of protein were loaded. (+) indicates cells transduced with each siRNA construct, and (-) indicates cells transduced with plasmids containing control siRNA. WT = SW480 WT cells, 1K = SW480 1K cells, 2K = SW480 2K cells, 1K2K = SW480 1K2K cells.

Figure 2. *In vitro* analyses of SW480 cells expressing HIF-1 α and HIF-2 α siRNA

(a) Proliferation assay showing the difference in growth of SW480 cells. Mean cell counts in 3 independent wells; bars, SD. There is no significant difference in the proliferation rates between SW480 WT cells and SW480 2K cells whereas there is a significant difference between SW480 WT cells and SW480 1K cells ($\# p < 0.01$). (b) Down-regulation of HIF-2 α expression leads to increased soft agar growth in SW480 cells. Quantitative summary of a representative soft agar assay. Columns, mean colony counts; bars, SD. There are no significant differences between SW480 WT cells and SW480 1K cells whereas there is a significant difference between SW480 WT cells and SW480 2K cells ($\# p < 0.01$). (c) Down-regulation of HIF-1 α expression leads to decreased migratory ability of SW480 cells. There are significant

reductions in migration between SW480 WT cells and SW480 1K cells ($p < 0.01$). A photograph of stained cells that have migrated through the membrane is illustrated below. WT = SW480 WT cells, 1K= SW480 1K cells, 2K= SW480 H2K cells, 1K2K = SW480 1K2K cells.

Figure 3.

Growth of SW480 cells *in vivo*. (a) Tumor weight and (b) tumor volume of SW480 WT, SW480 1K, SW480 2K and SW480 1K2K xenografts. There are significant differences in the tumor growth between SW480 WT and SW480 2K cells ($\# p < 0.01$) SW480 WT and SW 480 1K cells ($\# p < 0.01$), and SW480 WT and SW480 1K2K cells ($\# p < 0.01$). (c) Gross appearance (above) and H & E staining (below) of excised tumors at 6 weeks. (d) VEGF protein levels measured by ELISA. There are significant reductions in VEGF levels in SW480 1K, SW480 2K, and SW480 1K2K xenografts compared to SW480 WT tumors ($\# p < 0.01$). (e) Quantification of the microvessel density in SW480 xenografts. There are significant reductions in the microvessel density in the SW480 1K ($* p < 0.05$), SW480 2K ($* p < 0.05$), and SW480 1K2K ($\# p < 0.01$) xenografts compared with SW480 WT tumors. (f) Rates of PCNA positivity. There is a significant increase in the percent of PCNA positive cells between SW480WT and SW480 2K tumors ($\# p < 0.01$). WT = SW480 WT cells, 1K= SW480 1K cells, 2K= SW480 2K cells, 1K2K = SW480 1K2K cells.

Figure 4. Immunohistochemistry for HIF-1 α , HIF-2 α , CD31 and VEGF in representative human colon tumors. Both nuclear and cytoplasmic staining of HIF-1 α and HIF-2 α was observed whereas staining for VEGF was primarily cytoplasmic.

Figure 5.

(a) Expression profiles of 830 genes induced at least 2-fold by exposure to hypoxia in SW480 cells. Probes were classified into 4 groups based on their response to HIF1 α and HIF2 α siRNAs.
 (b) Venn diagram summarizing the 4 categories of hypoxia inducible genes.

Figure 6.

Validation of the expression levels of the HIF-2 α dependent genes, Cyclin G2 and Angiopoietin-like 4, by quantitative RT-PCR (qPCR). The microarray data are illustrated on the left and the qPCR data on the right. Expression levels in SW480 WT cells in normoxia are normalized to 1. W = SW480 WT cells, K1 = SW480 1K cells, K2 = SW480 2K cells, N = normoxia, H = hypoxia.

TABLE I
PATIENTS AND TUMOR CHARACTERISTICS

Characteristic	No. of patients (%)
Gender	
Male	26 (41.3%)
Female	37 (58.7%)
HIF-1 α staining	
Strong (>1+)	36 (57.1%)
Weak ($\leq$ 1+)	27 (42.9%)
HIF-2 α staining	
Strong (>1+)	28 (44.4%)
Weak ($\leq$ 1+)	35 (55.6%)
Tumor classification	
T1	4 (6.3%)
T2	17 (27.0%)
T3	33 (52.4%)
T4	9 (14.2%)
Lymph node status	
N0	36 (57.1%)
N1	16 (25.4%)
N2	11 (17.5%)
Stage	
I	16 (25.4%)
II	19 (30.2%)
III	22 (34.9%)
IV	6 (9.5%)

TABLE II
EXPRESSION OF HIF-1 α IN RELATION TO VEGF

		HIF-1α	
		Weak ($\leq 1+$)	Strong ($> 1+$)
VEGF	0	0	0
	1+–2+	7 (63.6%)	4 (36.4%)
	2+	19 (59.3%)	13 (40.7%)
	2+–3+	1 (5.0%)	19 (95.0%)

$p < 0.001$; Fisher's Exact Test (2×4).

TABLE III
EXPRESSION OF HIF-2 α IN RELATION TO VEGF

		HIF-2 α	
		Weak ($\leq 1+$)	Strong ($> 1+$)
VEGF	0	0	0
	1+–2+	9 (81.8%)	2 (18.2%)
	2+	18 (56.3%)	14 (43.7%)
	2+–3+	8 (40.0%)	12 (60.0%)

$p = 0.08$; Fisher's Exact Test (2×4).

TABLE IV
 EXPRESSION OF HIF-1 α IN RELATION TO MICROVESSEL DENSITY

		HIF-1 α	
		Weak ($\leq 1+$)	Strong ($> 1+$)
MVD	<10	5 (71.4%)	2 (28.6%)
	10–15	18 (58.0%)	13 (42.0%)
	>15	5 (18.5%)	22 (81.5%)

$p = 0.001$; Fisher's Exact Test (2×3).

TABLE V
EXPRESSION OF HIF-2 α IN RELATION TO MICROVESSEL DENSITY

		HIF-2α	
		Weak ($\leq 1+$)	Strong ($> 1+$)
MVD	<10	6 (85.7%)	1 (14.3%)
	10–15	17 (56.6%)	13 (43.4%)
	>15	12 (46.1%)	14 (53.9%)

$p = 0.18$; Fisher's Exact Test (2×3).

TABLE VI
EXPRESSION OF HIF-1 α IN RELATION TO TUMOR STAGE

		HIF-1α	
		Weak ($\leq 1+$)	Strong ($> 1+$)
Stage	1/2	16 (43.7%)	19 (54.3%)
	3/4	11 (49.3%)	17 (60.7%)

$p = 0.400$; Fisher's Exact Test (2×2).

TABLE VII
EXPRESSION OF HIF-2 α IN RELATION TO TUMOR STAGE

		HIF-2α	
		Weak ($\leq 1+$)	Strong ($> 1+$)
Stage	1/2	14 (40.0%)	21 (60.0%)
	3/4	21 (75.0%)	7 (25.0%)

$p = 0.004$; Fisher's Exact Test (2×2).

TABLE VIII
COMBINED EXPRESSION OF HIF-1 α AND HIF-2 α IN RELATION TO TUMOR STAGE

Group	A	B	C	D
	HIF-1 α weak HIF-2 α weak	HIF-1 α weak HIF-2 α strong	HIF-1 α strong HIF-2 α weak	HIF-1 α strong HIF-2 α strong
Stage ½	9 (50.0%)	7 (77.8%)	5 (29.4%)	14 (73.7%)
Stage ¾	9 (50.0%)	2 (22.2%)	12 (70.6%)	5 (26.3%)
Total	18	9	17	19

B vs. C; $p = 0.02$; Fisher's Exact Test (2×2).

C vs. D; $p = 0.02$; Fisher's Exact Test (2×2).