

Original Research Article

**Effect of hypoxia-inducible factor-1 and 2 on
tumor growth**

Abstract:

One of the well-known phenomenons that cause cancer is hypoxia, which has two forms: HIF-1 α and HIF-2 α . It is well known that both forms have high homology. In particular, HIF-1 α stimulates glycolytic gene expression in both types of cells clearly showing for the HIF-1 α and HIF-2 α (or HIF-1 and HIF-2) have singular aims; however, growing proof distinguishes between both molecules in terms of transcriptional targets and effect on different physiological pathways and tumorigenesis. Moreover, more interest has been paid in order to understand the real effect of HIF-2 α in order to control/regulate different sides of digestive-tumors; including angiogenesis, proliferation and apoptosis, metastasis, metabolism and resistance to chemotherapy. This makes HIF-2 α plays an essential and crucial role as regulator of the malignant phenotype. In the present study, one shows the importance role played by both HIF-1 and 2 through tumor growth.

Key words: HIF-1 α and HIF-2 α , tumor growth, malignant phenotype, regulated resistance,

Introduction

HIF-2 α is known as the 2nd organ of the hypoxia-inducible factor (HIF) family containing the well-known three proteins HIF1, HIF2 and HIF3. The amino-acid sequence of HIF-2 α is compatible with HIF1 to about 48% [1-3]. In addition, the super

family of transcription factors bHLH-PAS with HIF-2 α and HIF-1 β are the roots of HIF-2 α and HIF-1 β . HIF-2 α activates and relates transcription factors bHLH-PAS. It is, also, known as endothelial PAS domain protein-1 (EPAS1) [4]. PHDs introduce hydroxyl groups in order to yield two 2-proline residues: Pro 405 and 503 through HIF-2 α . This occurs by the aid of the cofactors O₂, Fe and α -ketoglutarate. Decreasing the biochemical-activity of PHD, HIF-2 α get in the nucleus and hetero-dimerized with HIF-1 β . Thereafter, HIF-2 α joins to a conserved DNA sequence referred as HRE "the hypoxia-response element"; then it Trans-activates a variety of HRGs "hypoxia-responsive genes"[5]. Even though HIF-2 α . shows stability at high oxygen-pressure compared with HIF-1 α (in vitro), it has never been revealed under normoxic conditions in various organs [6-8]. While HIF-2 α is highly induced with hypoxia in all studied-organs such as lung, liver, pancreas, brain, heart and intestine [6-8]. Most cancer growth reveals hypoxia as common event. Most of tumors and their corresponding metastases contain either HIF-2 α or HIF-1 α , or they can be present both [9-12]. In addition, bHLH-PAS is involved another protein in carrying out hypoxia response [13-16]. In addition, from structure point of view, about 48% of the amino acid prints in HIF-1 α , HIF-2 α are similar with 83% similarities in their bHLH domains [13- 16] and they have certain symmetric level in their O₂ dependent degradation domains. Moreover, the fifty amino acids groups situated "C" ends of HIF-1 α , HIF-2 α are strongly protected and show signs to improve the mechanism for oxygen regulation interactions with transcriptional-family p300 [17- 23].

2. Materials and Methods

2.1. Cell culture

Under the Uniform Biological Materials Transfer Agreement (UBMTA), Addgene, Suite 550A, Cambridge, MA 02139, USA, have provided 786-O renal carcinoma cells. The cells infected with free nucleic acid (pcDNA3-hygromycin vector (WT-8V)). In addition, due to the presence of free nucleic acid (pcDNA3-HA-VHL (RCC-4T 3-14)), we choose RCC-4 renal carcinoma RCC-4 (renal carcinoma cells) infected in our study. This presence of HepG2-cells, with HEK293, and Hep3B-cells helped culturing-processes under 1.5% oxygen in the presence of 1% fetal calf serum with 4.5 g of glucose/ml, 25 mM HEPES, for hypoxia or 21% O₂ for normoxia experiments. Human micro vascular endothelial cells lung were provided from Clonetics (Walkersville, Md., USA). The provider suggested growing conditions in EGM-2MV Bulletkit purchased from Lonza Walkersville, Inc, USA.

2.2. Preparation, detection and quantification of RNA

Following Streit et al [24], with the presence of TRIzol reagent, the Northern blot studies have been carried out conforming to standard protocols [25]. With the aid of using accession number from GenBank, different nucleotide (nt) have assessed the human DNA fragments. We carried out this with using reverse transcription-PCR and cloned into pCRII-topo vector to serve as templates.

2.3. Arranging of c-RNA and DNA microarray

We cultivated the above mentioned 786-O WT-8 cells 21% oxygen for one day; then

triplicate RNA-groups were arranged in order to initiate the DNA-microarray analyses. From total RNA with the Super- script Choice system (Gibco BRL Life Technologies) and T7-(dT)24 primers, one generates cDNA. In addition, with a BioArray high-yield RNA transcript labeling kit (Enzo Diagnostic, Inc.) biotin-labeled ribonucleotides were synthesized. In order to examine the fragmented cRNA for quality, we carried out two tests: First, by a test array and then, second, it was subjected to hybridization with human genome U95A arrays containing approximately 12,000 known mRNA transcripts (Affymetrix). Gene-chip expression analysis software was used to get complete analysis of data. First, individual samples were analyzed for absolute call and average differentiation. Then gene expression levels from two samples (normoxia and hypoxia) were subjected to comparative analysis for all three sets of data.

Analysis of HIF-1, HIF-2, and ARNT using preparation by nuclear extract and Western blot.

In the presence of proteinase inhibitors, we used 200 M deferoxamine (DFX) to prepare nuclear extracts.

Comparable amounts of protein as determined by Bradford protein assay (BioRad) were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blockage in Tris-buffered saline containing 5% nonfat milk, membranes were probed with anti-HIF-1 monoclonal antibody (NB 100-105; Novus Biological, Inc), anti-HIF-2 poly- clonal antibody (NB 100-122; Novus), or anti-ARNT monoclonal antibody (NB 100-124; Novus) in Tris-buffered saline with 3% nonfat milk. Horseradish per- oxidase-conjugated goat

anti-rabbit immunoglobulin G for polyclonal primary antibody or horseradish peroxidase-linked anti-mouse immunoglobulin G for monoclonal primary antibody in combination with Super Signal Chemiluminescent Substrate were used for signal detection.

Structure of Plasmid

Full-length murine HIF-1 or HIF-2 cDNAs were generated by reverse transcription-PCR and by inserting into a pcDNA3 expression vector. This happened by using primers incorporated with restriction sites.

For HIF-1	5-ATAggatcc(<i>Bam</i> HI) <u>ATG</u> GAGGGCGCCGGCGGCGAGA AC-3 (sense)
	5 -CGCctcgag(<i>Xho</i> I) <u>TCAG</u> TTA ACTTGATCCAAAGCTCT G-3(antisense).
For HIF-2	5-CATggtacc(<i>Kpn</i> I)CGGC GACA <u>ATG</u> ACAGCTGACAAGGAG-3 (sense)
	5 -CATtctaga(<i>Xba</i> I) <u>TCA</u> GGTGGCCTGGTCCAGAGCTCTGAG-3 (antisense)

Underlined letters indicate the start codon (ATG) and stop codon (TCA) of HIF-1 and HIF-2. A 10-amino-acid *c-myc* tag was further inserted at the C terminus of both HIF-cDNAs by using a *Pfu*-PCR-based protocol. For HIF-proline mutants, the same *Pfu*-PCR protocol was used to change proline residues to alanines by amplifying the full-length plasmid with primers incorporated with mutation (indicated in lowercase) as follows:

For HIF-1	P402A, 5-GCTGCCGGCGACACCATCATCTCTC-3
	5-g gcAGCCAGCAGAGTGAGAGCATCAGGC-
For HIF-2	P530A, 5-gcc-TAC ATCCCTATGGACGGCGAGG-3

	5-TGCCAAGGTCTCCAAATCCAG TTC-3
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Construction of HEK293 TET-on HIF- DPA clones.

Full-length cDNA of HIF-1 mycDPA (double prolines to alanines) or HIF-2 mycDPA in pcDNA. was amplified and inserted into a TET response element (TRE) vector (pTRE2hyg; Clontech) by using the following primer pairs:

For HIF-1	5 - ACCAacgcgt(<i>Mlu</i> I)CCGCCATGGAGGGCGCCGGCGGCGAGAAC-3
	5 - ACCAgcggccgc(<i>Not</i> I)TCACAGATCCTCTTCTGAGATGAG-3
For HIF-2	5 - ACCAgcggccgc(<i>Not</i> I)TCACAGATCCTCTTCTGAGATGAG-3
	5 -ACCAacgcgt(<i>Mlu</i> I)CCGCCATGACAGCTGACAAGGAGAAAAAAG G-3

HEK293 TET-on HIF- DPA clones were constructed by transfection of HEK293 TET-on cells (Clontech) with pTRE2hygHIF-1 DPA or pTRE2hygHIF-2 DPA in addition to pTet-tTS vector in the presence of 50 μ g of hygromycin/ml and 100 μ g of G418/ml.

Transient transfection.

Transfection of HEK293 cells with reporter and HIF- expression plasmid vectors was performed with Fugene 6 (Roche) according to manufacturer's instructions. The wild-type HRE-luciferase reporter as well as a mutant HRE-luciferase reporter were previously described [26]. A total of 50 to 60% confluent HEK293 cells in 6-well plates were transfected with 200 ng of reporter, 200 ng of expression plasmid, and 100

ng of reference plasmid murine sarcoma virus-galactosidase. One transfected them for 40 hours transfection, and collect them in buffer (Promega). Then we assayed them for luciferase activity as a function of HIF- and -galactosidase activity.

Glucose assay and VEGF enzyme-linked immunosorbent assay

For glucose concentration and VEGF protein secretion assays, the same number of HEK293

TET-on HIF-1 DPA or HEK293 TET-on HIF-2 DPA or HEK293 TET-on parental cells were grown in high-glucose Dulbecco's modified Eagle's medium (4.5 g/ml) with (1 g/ml) or without doxycycline. A total of 400 μ l of the medium was collected for the glucose and VEGF assay at indicated times. The amount of glucose was quantitated with Sigma Diagnostics glucose reagents. VEGF quantitation was performed with a QuantiKine human VEGF immunoassay kit (R&D Systems).

RESULTS

Hypoxia-inducible genes in 786-O WT-8 cells.

Compernelle et al [27] have reported that both HIF-1 and HIF-2 have been implicated in hypoxia responses [27]. However, their relative contributions to hypoxic gene induction are unknown, since most O₂-regulated genes have been identified in cells that express both HIF-1 and HIF-2. A well-studied cell line, 786-O WT-8, was chosen to evaluate HIF-2 target genes [28-30]. 786-O cells were originally isolated from patients with renal clear cell carcinoma and exhibited mutated *VHL*. Stable transfection with either pRc/CMV empty vector or pRc/CMV-HA-tagged *VHL* gave rise to 786-O PRC-3 and 786-O WT-8, respectively [28, 29]. Northern blot analysis of total RNA from

786-O cells detected no HIF-1 mRNA (Fig. 1, lanes 1 to 4), a result that is consistent with previous reports [30].

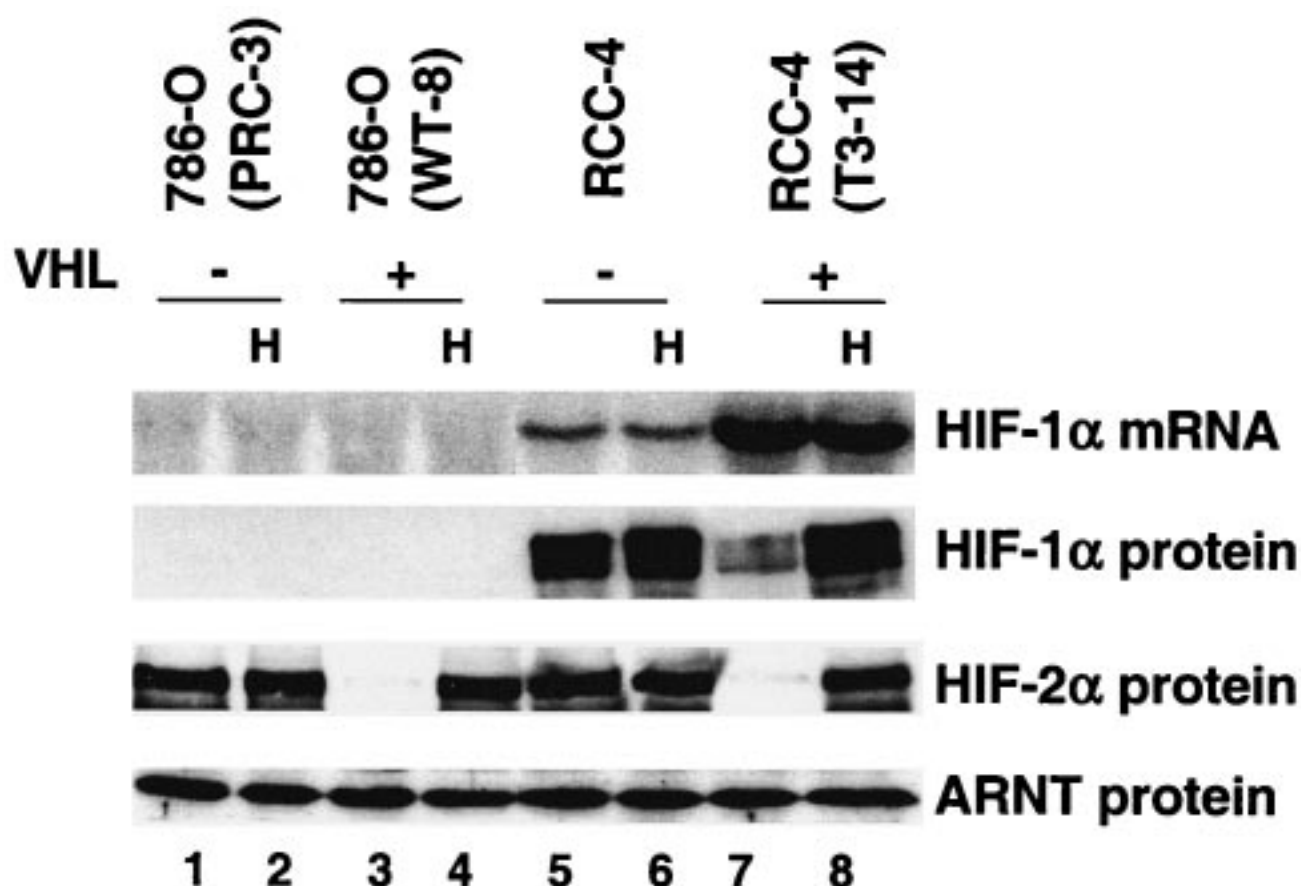


FIG. 1. 786-O cells express HIF-2 but not HIF-1. 786-O (PRC-3) is a *VHL*-defective renal carcinoma cell line, whereas 786-O (WT-8) cells have been stably transfected with functional *VHL* (38). RCC-4 is another *VHL*-mutant renal carcinoma cell line, expressing both HIF-1 and HIF-2. The RCC-4 (T3-14) cells have been stably transfected with functional *VHL*. Enhanced expression of HIF-1 mRNA in *VHL*-transfected RCC-4 cells has previously been observed (55). Hypoxia (H) treatment (1.5% O₂ for 16 h) stabilized HIF-2 protein in 786-O (WT-8) cells and stabilized both HIF-1 and HIF-2 in RCC-4 (T3-14) cells as shown by HIF-1 and HIF-2 Western blots. The ARNT Western blot served as a protein loading control

Importantly, no HIF-1 protein was detected even in the *VHL*-defective 786-O PRC-3 cells (Fig. 1, lanes 1 and 2). However, 786-O cells do express HIF-2 protein. Consistent with their *VHL* status, 786-O PRC-3 cells grown at both 21 or 1.5% O₂ expressed similar levels of HIF-2 (Fig. 1, lanes 1 and 2), but 786-O WT-8 cells

exhibited O₂-dependent regulation of HIF-2 protein (Fig. 1, lanes 3 and 4). Another *VHL*-defective renal carcinoma cell line, RCC-4, expresses both HIF-1 and HIF-2 proteins, independent of O₂ tension (Fig. 1, lanes 5 and 6). Stable transfection of pcDNA3-HA-tagged *VHL* (RCC-4T 3-14) reduced normoxic HIF- protein levels, restoring hypoxia inducibility (Fig. 1, lanes 7 and 8).

To systematically study HIF-2 -inducible genes, triplicate RNA samples were prepared from 786-O WT-8 cells cultured under normoxia (21% O₂) or hypoxia (1.5% O₂) for 16 h. Double-stranded cDNA was generated from total RNA with the Superscript Choice System (Gibco BRL Life Technologies) and T7-(dT)₂₄ primers. Subsequently, antisense biotin-labeled RNA was synthesized with T7 DNA polymerase. The RNA probe was fragmented and subjected to hybridization with human genome U95A arrays containing approximately 12,000 known mRNA transcripts (Affymetrix). A comparative analysis of genes expressed under hypoxia versus normoxia indicated that 1.5% O₂ treatment stimulated 30 genes in the triplicate RNA samples. However, expression of some genes is reduced by hypoxia (data not shown). The induced genes included novel hypoxia-inducible genes such as those for ADRP, chemokine growth-regulated oncogene2 (GRO-2), baculoviral inhibitor of apoptosis (IAP) repeat-containing protein 3 (BIRC-3; also called cIAP-2), and known HIF target genes encoding VEGF and ADM in 786-O cells. However, another known HIF target in 786-O cells, platelet-derived growth factor, B chain (PDGF-B), is not on the human genome U95A arrays. ADRP protein is frequently found at the surface of lipid droplet (the spherical organelles for neutral lipid storage) and has been shown to promote lipid accumulation and lipid droplet formation [31, 32]. Its overexpression in *VHL*- defective

renal clear cell carcinoma may explain the clear cell phenotype, a phenomenon that arises when a large amount of lipid droplets in such cells is removed during sample preparation for microscopy.

Some of these hypoxia-inducible genes identified by DNA microarray assays were further tested, and all those tested were confirmed by Northern blot analysis in triplicate RNA samples, including VEGF, which exhibited a 1.4-fold induction in the DNA microarrays. Since the expression pattern of these genes was identical in all three sets of RNA samples, only one set of Northern blotting results is presented. Figure 2A shows the results for several genes, likely to be HIF-2 targets, including a novel hypoxia-inducible gene, the ADRP gene (induction, 2.6-fold based on phosphorimager analysis of Northern blots), and three known hypoxia-inducible genes, NDRG-1 (2.0-fold), ADM (1.8-fold), and VEGF (1.8-fold) genes (Fig. 2A, lane 4 versus lane 3). To separate HIF-dependent transcription stimulation from other hypoxia effects, such as hypoxia-induced mRNA stability [33- 35] or hypoxia-induced AP-1 (4, 5) and NF-B activity [36, 37], target gene expression was also assessed in otherwise genetically identical *VHL*-defective 786-O PRC-3 cells. Interestingly, high levels of gene expression were observed in 786-O PRC cells, independent of O₂ tension (Fig. 2A, lanes 1 and 2 versus lane 3). The target gene expression (Fig. 2A) clearly correlated with HIF-2 protein levels (Fig. 1), suggesting that HIF-2, and no other hypoxia-stimulated transcription factors such as AP-1 and NF-B, activates the expression of these genes. ADRP (induction, 2.5-fold), NDRG-1 (2.7-fold), ADM (2.9-fold), and VEGF (2.4-fold) were also stimulated by hypoxic treatment of RCC-4T 3-14 (with functional pVHL) (Fig. 2A, lane 9 versus lane 8) and their expression levels correlated

well with HIF- protein levels (Fig. 1, lanes 5 to 8). However, we could not confirm which HIF-subunit plays a role in RCC-4 gene activation, as RCC-4 cells express both HIF-1 and HIF-2. The expression of GADD45A (induction, 1.9-fold) and GADD153 (also called CHOP; induction, 1.8-fold) were also induced by low O₂ (Fig. 2B, lane 4 versus lane 3) as previously reported [38]. However, in contrast to the expression of the above-described genes, the expression of GADD45A and CHOP appears to be independent of the HIF-/VHL pathway, since their expression did not correlate with HIF-2 protein levels.

In conclusion, analysis of HIF-2 target genes by DNA microarray and Northern blot analysis in *VHL*-defective cells as well as in cells with functional *VHL* demonstrated that HIF-2 is active in 786-O cells. Moreover, several novel functionally important genes were identified (see Discussion).

HIF-2 is not involved in regulating the glycolytic pathway.

As shown by numerous reports, genes involved in glucose metabolism are ubiquitously expressed and stimulated by hypoxia [39- 41]. With similar DNA microarray analyses, most glycolytic genes were induced in hypoxia-treated wild-type murine ES cells. Surprisingly, no glycolytic gene induction was observed in O₂-deprived 786-O WT-8 cells, although all the glycolytic genes are present on the human genome U95A DNA microarray. The expression of 13 glycolytic genes was individually tested by Northern blot analysis, confirming the DNA microarray data indicating that these genes are not induced by hypoxia in 786-O WT-8 cells (Fig. 2C, lane 4 versus lane 3; also data not shown). Figure 2C shows the results of four representative genes, including two strongly hypoxia-responsive genes, corresponding to PGK-1 and LDHA, and two

moderately hypoxia-responsive genes, PGM-1 and PKM. Expression of the four genes was not responsive to hypoxia treatment in 786-O WT-8 cells (Fig. 2C, lane 4 versus 3).

Severe hypoxia treatment (0.1% O₂) also failed to enhance the expression of these particular genes (Fig. 2C, lane 5 versus lane 3).

In contrast, elevated induction at 0.1% O₂ was observed for HIF-2-responsive genes, such as those for ADRP (3.5-fold), NDRG-1 (6.6-fold), ADM (3.7-fold), and VEGF (3.1-fold) (Fig. 2A, lane 5 versus 3). It is noteworthy that the normoxic level of glycolytic genes in 786-O WT-8 cells was comparable to that in *VHL*-defective parental 786-O PRC-3 cells (Fig. 2C, lane 1 versus lane 3), indicating that HIF-2 had no effect on their expression. In contrast, O₂-deprived RCC-4T 3-14 cells (which express both HIF-1 and HIF-2) exhibited hypoxic induction of PGK (2.5-fold), LDHA (3.0 fold), PGM-1 (1.6-fold), and PKM (1.5-fold) gene expression in parallel experiments (Fig. 2C, lane 9 versus lane 8). The glycolytic genes also appeared to be direct targets of HIF, since *VHL* status clearly affected the expression level of each gene (Figure C, lanes 6 to 9).

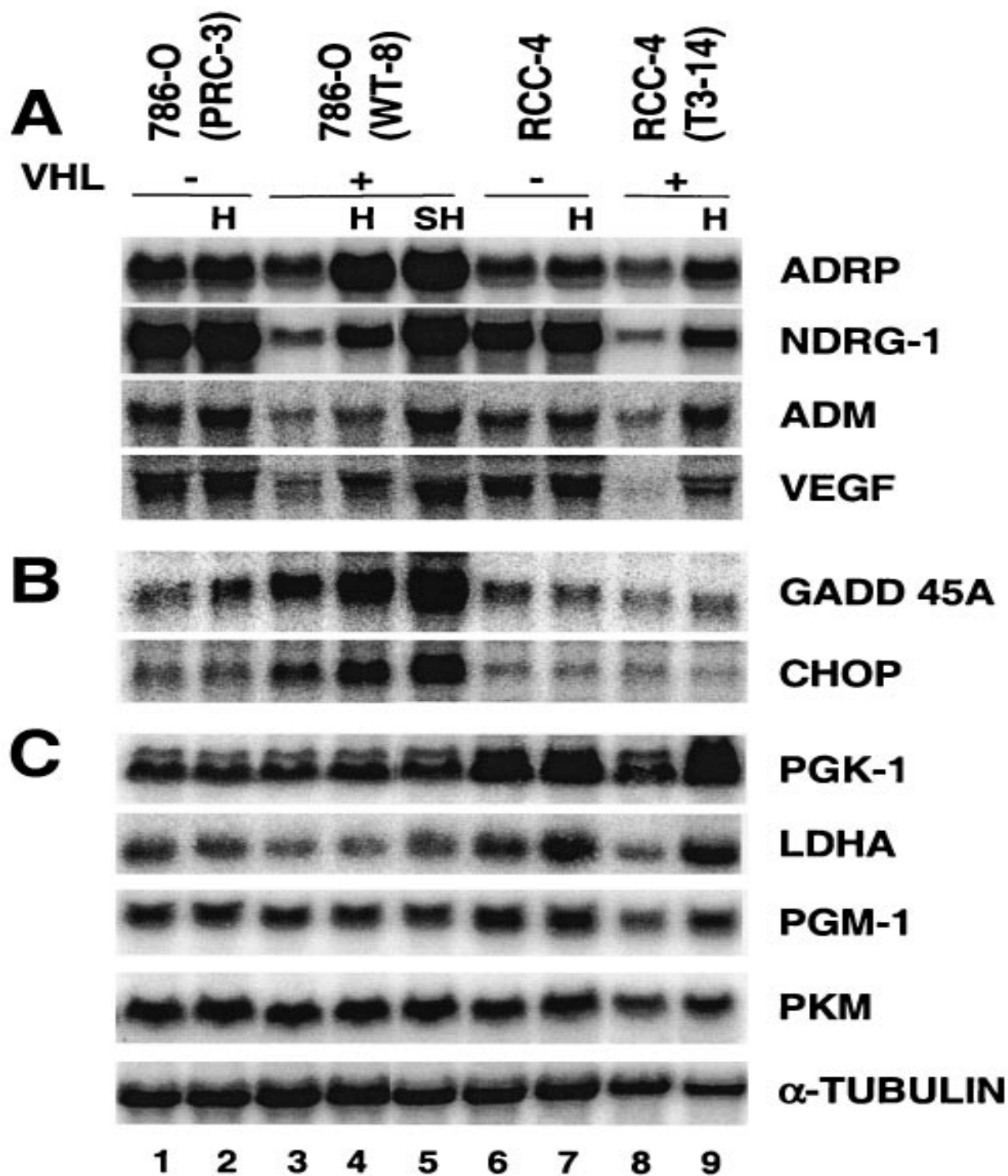


FIG. 2. Confirmation of DNA microarray data by Northern blot analyses. (A) Hypoxia (H) (1.5% O₂ for 16 h) stimulates expression of ADRP, NDRG-1, ADM, and VEGF in both 786-O (WT-8) and RCC-4 (T3-14) cells and is likely to be HIF-dependent since these genes are overexpressed in the parental 786-O (PRC-3) and RCC-4 cells independent of O₂. Severe hypoxia (SH) (0.1% O₂ for 16 h) further

enhances hypoxic gene expression in comparison to 1.5% O₂. (B) GADD45A and CHOP are hypoxia-responsive genes but are independent of HIF activity. (C) PGK-1, LDHA, PGM-1, and PKM mRNAs, which encode four glycolytic enzymes, are not stimulated by hypoxic treatment (1.5 or 0.1% O₂) of 786-O cells but are induced in RCC-4 cells.

Associating HIF-1 with up-regulation of glycolytic genes in multiple cell types.

The presence of HIF-1 in RCC-4 cells and the absence of HIF-1 in 786-O cells may explain why glycolytic genes were induced by hypoxia only in RCC-4 cells. To investigate the connection between HIF-1 and hypoxic glycolytic gene induction, we surveyed HIF-1 and HIF-2 expression and glycolytic gene inducibility in multiple cell lines. HUVECs and HMVEC-Ls are two primary cells from normal donors, while the remaining three cell lines, HEK293, HepG2, and Hep3B, are transformed. All five cell lines expressed similar levels of HIF-1 mRNA, while the two endothelial cells expressed higher levels of HIF-2 mRNA (Fig. 3A), a result that is consistent with a previous study showing that the levels of HIF-1 mRNA varied much less than that of HIF-2.

The levels of HIF-mRNA under hypoxia were slightly reduced relative to that under normoxia, indicating that transcription of HIF-1 and HIF-2 is not elevated by hypoxia. However, hypoxia-mimetic agents increased the levels of both HIF-1 and HIF-2 proteins in all five-cell types (Fig. 3B). In general, agreement with HIF-1 mRNA levels, all the five cell lines (except HMVEC-L) exhibited similar levels of HIF-1 protein. The much lower level of HIF-1 protein in HMVEC-L cells (primary cells from lung) is consistent with a previous study with similar HMEC-1 cells (simian virus 40 large T antigen transformed from dermal vessels) [42]. The two endothelial cells exhibited higher levels of HIF-2 protein than 293 and HepG2 cells expressed, in

keeping with their mRNA levels. In contrast, Hep3B cells expressed the highest levels of HIF-2 protein among all five cell lines although it expresses less HIF-2 mRNA than either endothelial cell line. Interestingly, different levels of hypoxic gene induction were observed in various cell lines. While high levels of glycolytic gene induction were exhibited by HepG2 (PGK, 7.5-fold; LDHA, 4.4-fold) and Hep3B cells (PGK, 4.5-fold; LDHA, 3.5-fold), low levels of induction were exhibited by HUVEC (PGK, 1.5-fold; LDHA, 1.8-fold), HMVEC-L (PGK, 1.5-fold; LDHA, 2.4-fold), and HEK293 cells (PGK, 2.5-fold; LDHA, 2.4-fold).

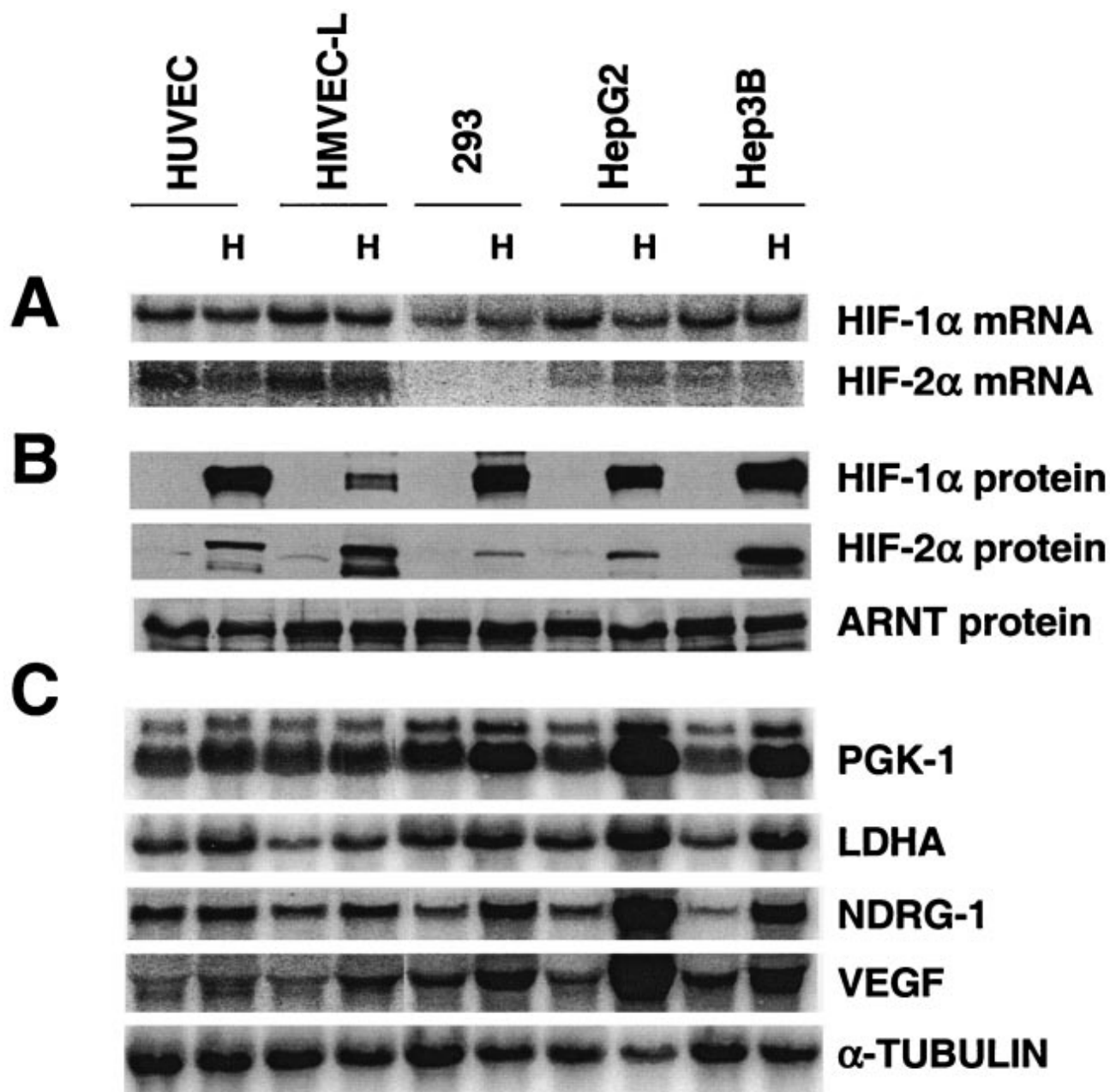


FIG. 3. HIF-1 is associated with hypoxic induction of glycolytic genes in multiple cell types. (A) Northern blot analysis of HIF-1 and HIF-2 expression in two primary endothelial cell lines and three transformed cell lines. HIF-1 is expressed in all five lines with similar levels, whereas HIF-2 is expressed highly in two endothelial cells. (B) Western blot analysis of HIF-1 and HIF-2 protein in cells treated with hypoxia-mimetic DFX (100 M) for 6 h. The levels of HIF-1 and HIF-2 protein expression are in general agreement with their mRNA levels, except for the levels of HIF-1 in HMVEC-L cells and HIF-2 in Hep3B cells. (C) Hypoxic treatment (1.5% O₂ for 16 h) increases the expression of the glycolytic genes (PGK and LDHA) and HIF-2 inducible genes (NDRG-1 and VEGF) in all five-cell lines.

However, this low level of induction is not specific for glycolytic genes. This is because HIF-2 inducible genes (NDRG-1 and VEGF genes) also exhibited low levels of induction in HUVECs (NDRG-1, 1.4-fold; VEGF, 1.6-fold), HMVEC-Ls (NDRG-1, 2.4-fold; VEGF, 2.4-fold), and HEK293s (NDRG-1, 2.8-fold; VEGF, 2.5-fold) and higher levels of induction in HepG2s (NDRG-1, 12-fold; VEGF, 9.5-fold) and Hep3Bs (NDRG-1, 6-fold; VEGF, 3.0-fold).

Cell type rather than the amount of HIF- may determine the level of hypoxic gene induction, since low levels of induction were observed in HUVEC-L cells expressing high levels of both HIF-1 and HIF-2. Importantly, all five HIF-1-expressing cells exhibited glycolytic gene induction under low O₂ levels, consistent with a role for HIF-1 in regulating glycolytic enzyme expression (Fig. 3C).

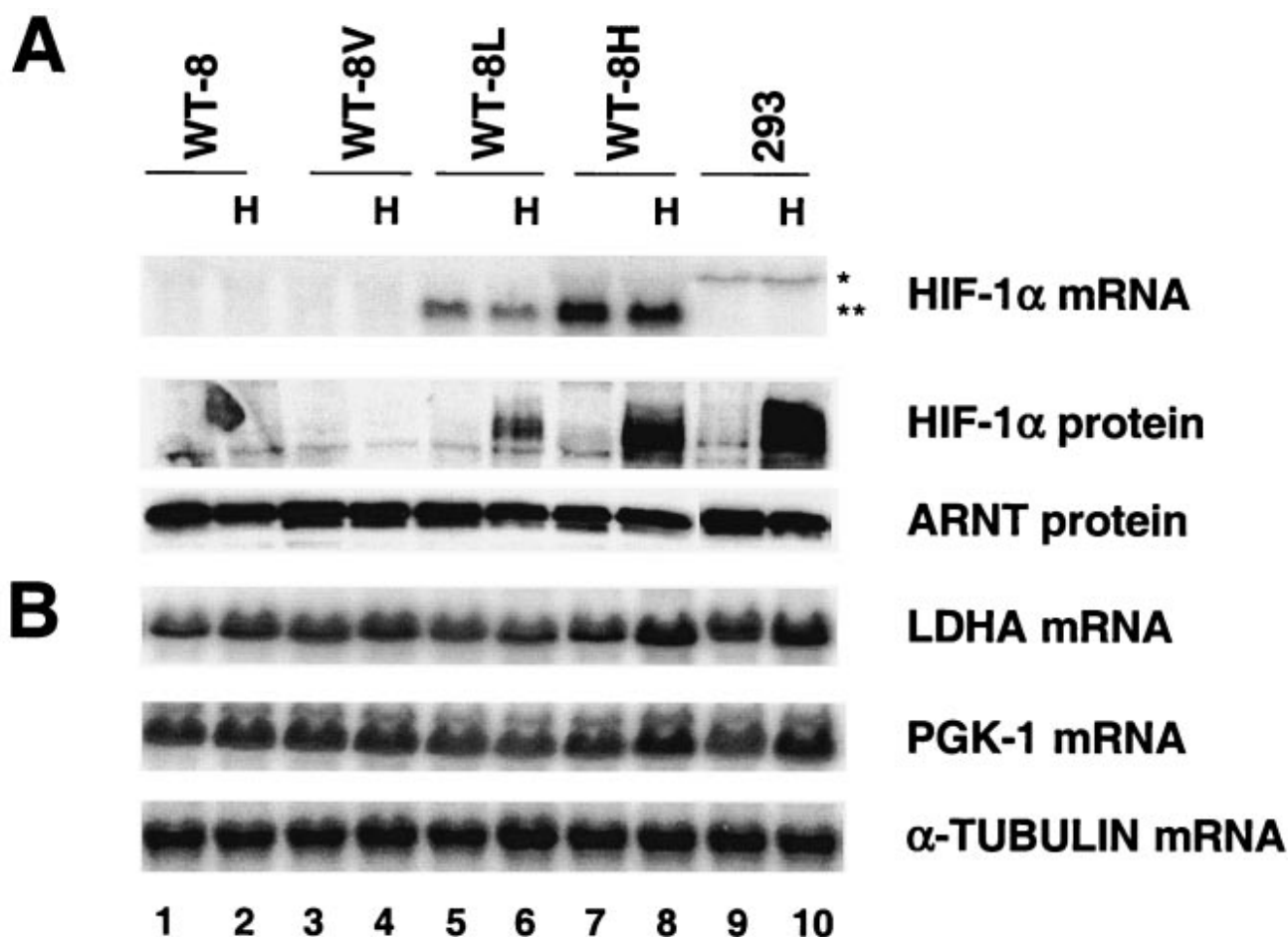


FIG. 4. HIF-1 expression in 786-O cells restores the glycolytic gene response to hypoxia treatment. (A) Establishment and characterization of 786-O (WT-8) clones stably transfected with HIF-1. WT-8L cells express low levels of HIF-1, whereas WT-8H cells express higher levels of HIF-1 as demonstrated by HIF-1 Northern blot and Western blot assays. The pcDNA3 vector-transfected control WT-8V cells and parental WT-8 cells express no HIF-1 RNA or protein. HEK293 cells, whose endogenous HIF-1 mRNA (asterisk) exhibits a larger size than transfected HIF-1 mRNA (double asterisk), served as positive control for HIF-1 protein. (B) Northern blot analysis of two glycolytic genes in HIF-1-rescued WT-8 cells, showing that LDHA and PGK-1 are induced in WT-8H cells after treatment with 1.5% O₂ for 16 h. The level of induction in WT-8H is comparable to that in HEK293 cells by endogenous HIF-1α.

Stable transfection of HIF-1 into 786-O WT-8 cells re-stores glycolytic gene induction.

To prove (formally) that a lack of HIF-1, and not genetic defects in glycolytic genes, leads to glycolytic gene unresponsiveness to hypoxic treatment of 786-O cells, we introduced wild-type murine HIF-1 cDNA into 786-O WT-8 cells and made stable

HIF-1 transfectants. As shown in Fig. 4A, WT-8L cells express low levels of transfected HIF-1 mRNA (Fig. 4A, lanes 5 and 6), whereas WT-8H cells express high levels of HIF-1 mRNA (Fig. 4A, lanes 7 and 8).

The empty vector-transfected WT-8V cells (Fig. 4A, lanes 3 and 4) and parental 786-O WT-8 cells (Fig. 4A, lanes 1 and 2) do not express HIF-1 mRNA. Although both WT-8L and WT-8H clones express detectable HIF-1 mRNA levels, no HIF-1 protein was detected under normal O₂ concentrations (Fig. 4A, lanes 5 and 7). Hypoxic treatment stabilized HIF-1 protein at levels correlating with mRNA levels (Fig. 4A, lanes 6 and 8) with WT-8H cells exhibiting a level of HIF-1 protein similar to that of control HEK293 cells under hypoxia (Fig. 4A, lane 10). Identical amounts of HIF-2 protein were detected in hypoxia-treated WT-8 and WT-8H cells, indicating that expression of HIF-1 had no effect on the level of HIF-2. Importantly, glycolytic genes such as those for LDHA and PGK-1 were induced in the HIF-1 transfectants (Fig. 4B). The level of induction also appeared to correlate with HIF-1 protein levels, as stronger induction was observed in the WT-8H cells (PGK, 1.7-fold; LDHA, 2.1-fold), which express higher levels of HIF-1 (Fig. 4B, lane 8 versus lane 7) and lower induction in the WT-8L cells (PGK, 1.3-fold; LDHA, 1.4-fold) (Fig. 4B, lane 6 versus lane 5). This level of induction in WT-8H cells is comparable to that seen in HEK293 cells (PGK, 1.9-fold; LDHA, 1.8-fold) (Fig. 4B, lane 10 versus lane 9). These results demonstrated that the absence of hypoxic glycolytic gene induction in 786-O WT-8 cells is due to the lack of HIF-1 in these cells, suggesting that HIF-1 is the only hypoxia-inducible factor able to target glycolytic gene expression.

Generation of cell lines expressing stabilized HIF-1 and HIF-2

Our data raise several important questions. (i) Is the inability of HIF-2 to regulate glycolytic genes a cell-specific event? (ii) Can HIF-1 also regulate the hypoxia-responsive genes identified in 786-O WT-8 cells, or are these hypoxia-inducible genes uniquely regulated by HIF-2? (iii) Is HIF-2 or some other factor responsible for the observed hypoxia response in 786-O cells? To address these questions, we employed a TET-on gene expression system in which the expression of HIF-1 or HIF-2 mRNA is regulated by the addition of doxycycline, allowing us to study individual functions of HIF-subunits. Expression of wild-type HIF- mRNA did not result in expression of the HIF- protein under normoxia, as expected (Fig. 4A). To stabilize transfected HIF-protein under normoxia, two hydroxylated prolines in both HIF-1 and HIF-2 oxygen-dependent-degradation domains [43- 45] were mutated to alanine (Fig. 5A).

One evaluated HIF-protein normoxic stability by comparing protein levels recovered from transfected cells with or without 100-MDFX treatment for 6h, using transient transfection and Western blot assays. This was carried out consistent with previous reports, HIF-1 P577A or HIF-2 P530A exhibited higher protein levels than wild-type HIF-under normoxia, but DFX treatment could still enhance protein levels of these single proline mutants [43, 44]. The double proline mutants exhibited even higher stability than single proline mutants and DFX lost its stabilizing effect (Fig. 5B) [45]. Importantly, double proline mutants functioned like the wild-type HIF-, as demonstrated by transactivation of the wild-type HRE-Luc reporter but not the mutant HRE-Luc reporter under normoxia (Fig. 5C). Higher activation of these reporters by HIF-proline mutants likely reflects their increased stability. The mutant (MT)-HRE-

Luc reporters are not regulated by HIF-1 or HIF-2. HIF-1 DPA and HIF-2 DPA cDNAs under transcriptional control of the TRE were stably transfected into HEK293 TET-on cells, which express the reverse TET-controlled trans-activator. To block background expression of transfected HIF- mRNA from these constructs, TET-controlled transcription silencer (tTS) was also cotransfected. In the absence of doxycycline, tTS binds to the TET operator (TET-O) DNA sequence in the TRE region, preventing HIF- expression. Addition of doxycycline dissociates tTS from the TET O sequence and activates transcription by the TET-controlled trans-activator.

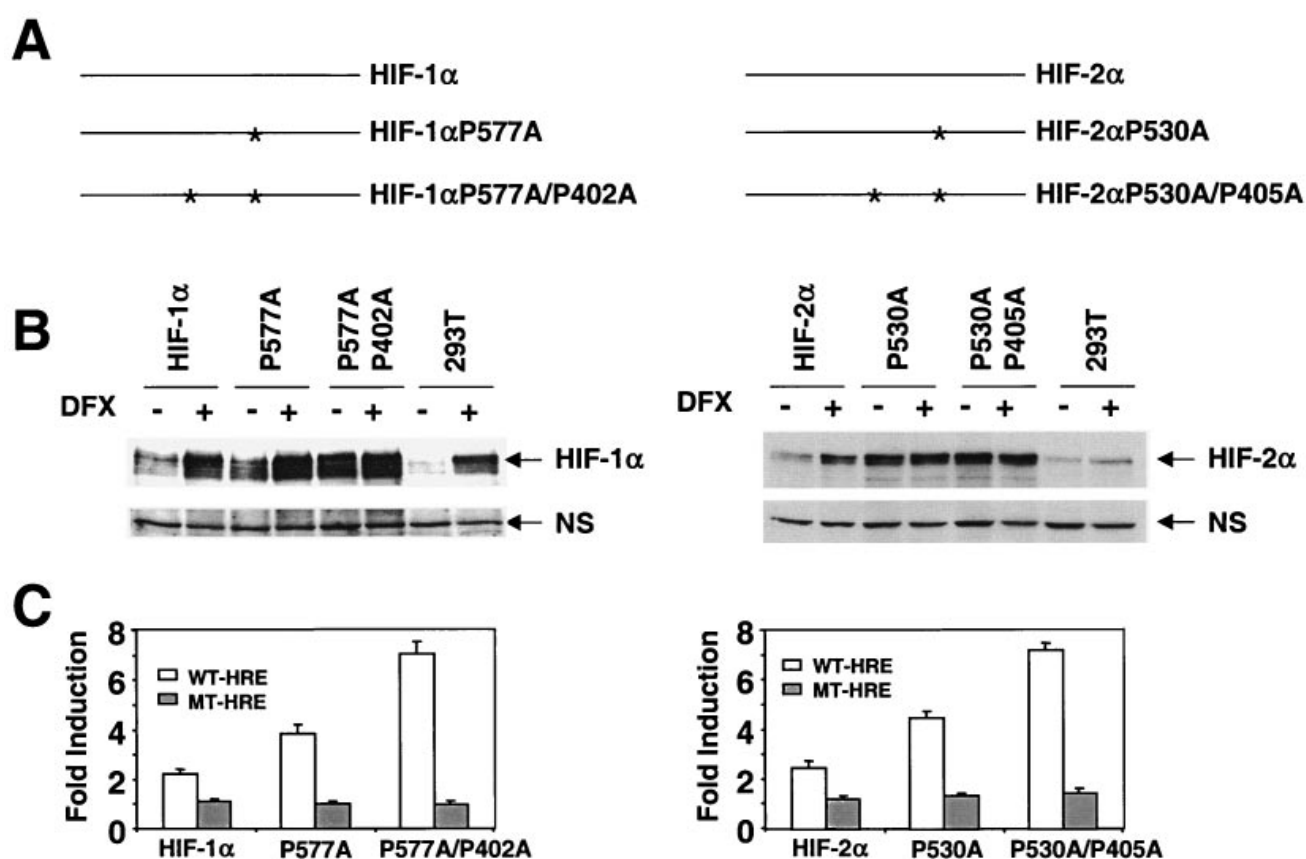


FIG. 5. Mutagenesis of hydroxylated prolines confers HIF-1 and HIF-2 function to normoxic cells. (A) Schematic representation of HIF-1 and HIF-2 single and double proline mutants. (B) Double proline HIF-1 and HIF-2 mutants are stabilized under normal O_2 tension. HEK293-T cells transfected with wild-type single-proline mutant and double-proline mutant HIF- subunits were split into two dishes, one of which was treated with DFX for 6 h before nuclear extraction preparation. The amount of HIF- protein was measured by Western blot analysis with specific antibodies and a nonspecific (NS) band is shown

for protein loading control. (C) HIF-double proline mutants are highly active in wild-type (WT)-HRE-Luc reporter transactivation assays in normoxic cells. The mutant (MT)-HRE-Luc reporters are not regulated by HIF-1 α or HIF-2 α

HEK293 TET-on HIF-1 DPA clones (Fig. 6A, left) and HIF-2 DPA clones (Fig. 6A, right) exhibited tight regulation of expression of transfected HIF-mRNA. No exogenous HIF-mRNA was detected without doxycycline. Transfected HIF-mRNA expression was induced when cells were treated with 0.1 g of doxycycline /ml and higher induction was obtained in a dose-dependent manner, which reached a plateau at 1 g of doxycycline/ml (Fig. 6A). However, expression of endogenous HIF-1 mRNA was not changed by exogenous HIF-1 mRNA (Fig. 6A left), verifying our experimental design that only exogenous HIF- function will be evaluated. The induction of HIF-mRNA by doxycycline occurred rapidly. HIF- mRNA was detected after cells were treated with 1 g of doxycycline/ml for 4 h and maximal levels were reached at 20 h, sustaining the high levels of HIF- mRNA for at least 48 h (data not shown). Importantly, the addition of doxycycline induced expression of not only HIF-mRNA but also the HIF-protein under normoxia, as demonstrated by Western blot analysis of HIF-proteins in nuclear extracts of two independent HEK293 TET-on HIF-1 DPA clones (Fig. 6B, left) and two independent HEK293 TET-on HIF-2 DPA clones (Fig. 6B, right). The establishment of HEK293 TET-on HIF- DPA clones allows assessment of HIF-1 and HIF-2 function in the absence of other hypoxic mechanisms.

HIF-1 is a master regulator of hypoxia responses.

Total RNA was harvested from two independent HEK293 TET-on HIF-1 DPA clones (130 and 357) and HEK293 TET-on parental cells, after treatment with (1g/ml) or

without doxycycline for 20 h under normal O₂ levels. The addition of doxycycline in HEK293 TET-on HIF-1 DPA clones 130 and 357 induced hypoxic gene expression, including PGK-1 (2.4- and 2.6-fold, respectively), LDHA (2.2- and 1.5-fold, respectively), NDRG-1 (2.6- and 3.6-fold, respectively), and VEGF (2.6 and 2.2-fold, respectively) (Fig. 7A). Other hypoxia-responsive genes, such as those corresponding to PGM-1, PKM, ALDA, ADRP, ADM, and GLUT-1, were also induced by HIF-1 in HEK293 TET-on HIF-1 DPA cells (data not shown). Importantly, doxycycline had no effect on the expression of these genes in HEK293 TET-on parental cells (data not shown).

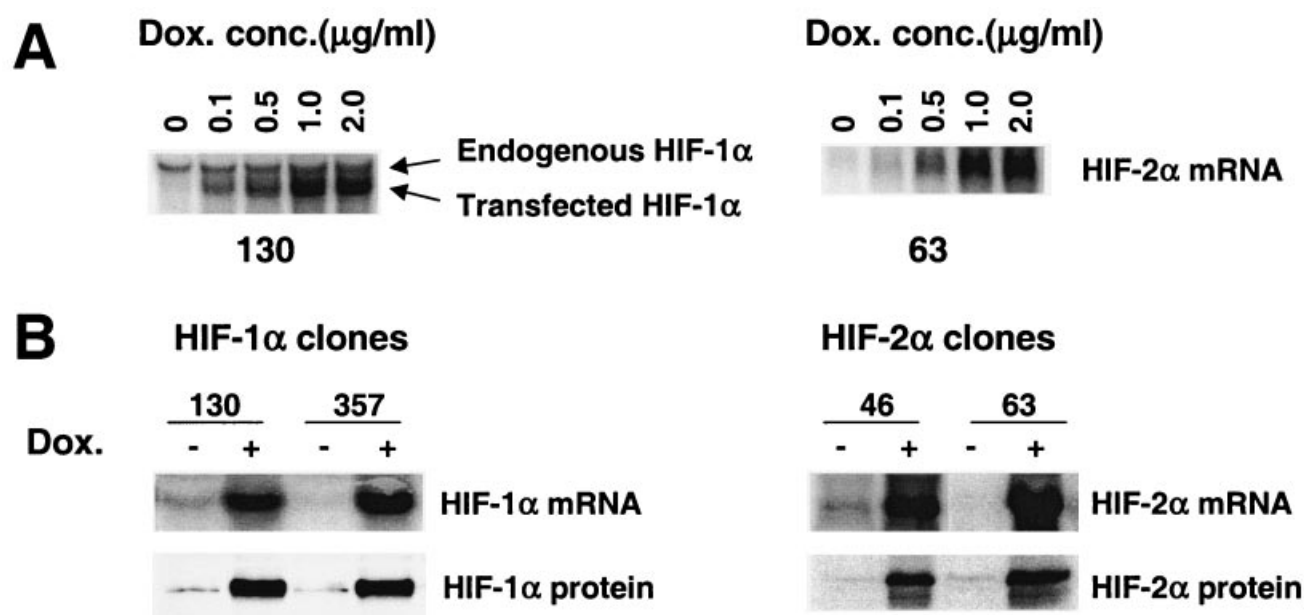


FIG. 6. Establishment of HEK293 TET-on HIF-1 DPA and HIF-2 DPA clones. (A) HIF-mRNA expression is tightly regulated by doxycycline. Northern blot analysis of HIF-1 and HIF-2 mRNA expression in HEK293 TET-on HIF-1 DPA clone 130 (left) and HEK293 TET-on HIF-2 DPA clone 63 (right). It was treated with different amounts of doxycycline for 20 h. (B) Doxycycline (1 μg/ml for 20 h) induced HIF-1 mRNA and protein expression in two independent HEK293 TET-on HIF-1 DPA clones (left) and HIF-2 mRNA and protein expression in two independent HEK293 TET-on HIF-2 DPA clones (right). Dox. conc. concentration of doxycycline.

hypoxia-inducible genes we have identified thus far. Interestingly, expression of the GADD45A and CHOP genes was not induced by HIF-1 (data not shown), confirming our previous observation in 786-O cells that hypoxic regulation of these genes is independent of HIF activity. Expression of some hypoxia-inducible genes was induced in 786-O cells (Fig. 2A). We assumed that these genes are regulated by HIF-2 and not by HIF-3 (or even unidentified HIFs) in 786-O cells. The HEK293 TET-on HIF-2 DPA clones allowed us to confirm that these genes are bona fide HIF-2 targets. Data in Figure 2 show that:

- (1) Expression of the HIF-2 DPA protein in clones 46 and 63 increased NDRG-1 (2.2- and 2.1-fold, respectively), VEGF (2.5- and 1.9-fold, respectively),
- (2) ADRP, ADM, and GLUT-1 expression (figure 7B and data not shown), consistent with the Northern blot data in Fig. 2. This demonstrates that HIF-2 is functional and capable of regulating these hypoxia-regulated genes in both HEK293 and 786-O cells. In contrast, HIF-2 did not activate PGK-1, LDHA, PGM-1, PKM and ALDA (Fig. 7B and data not shown), providing independent confirmation that HIF-2 does not transactivate glycolytic genes in another cell type. These data suggest that HIF-1 is a master hypoxic regulator and can activate all HIF-dependent

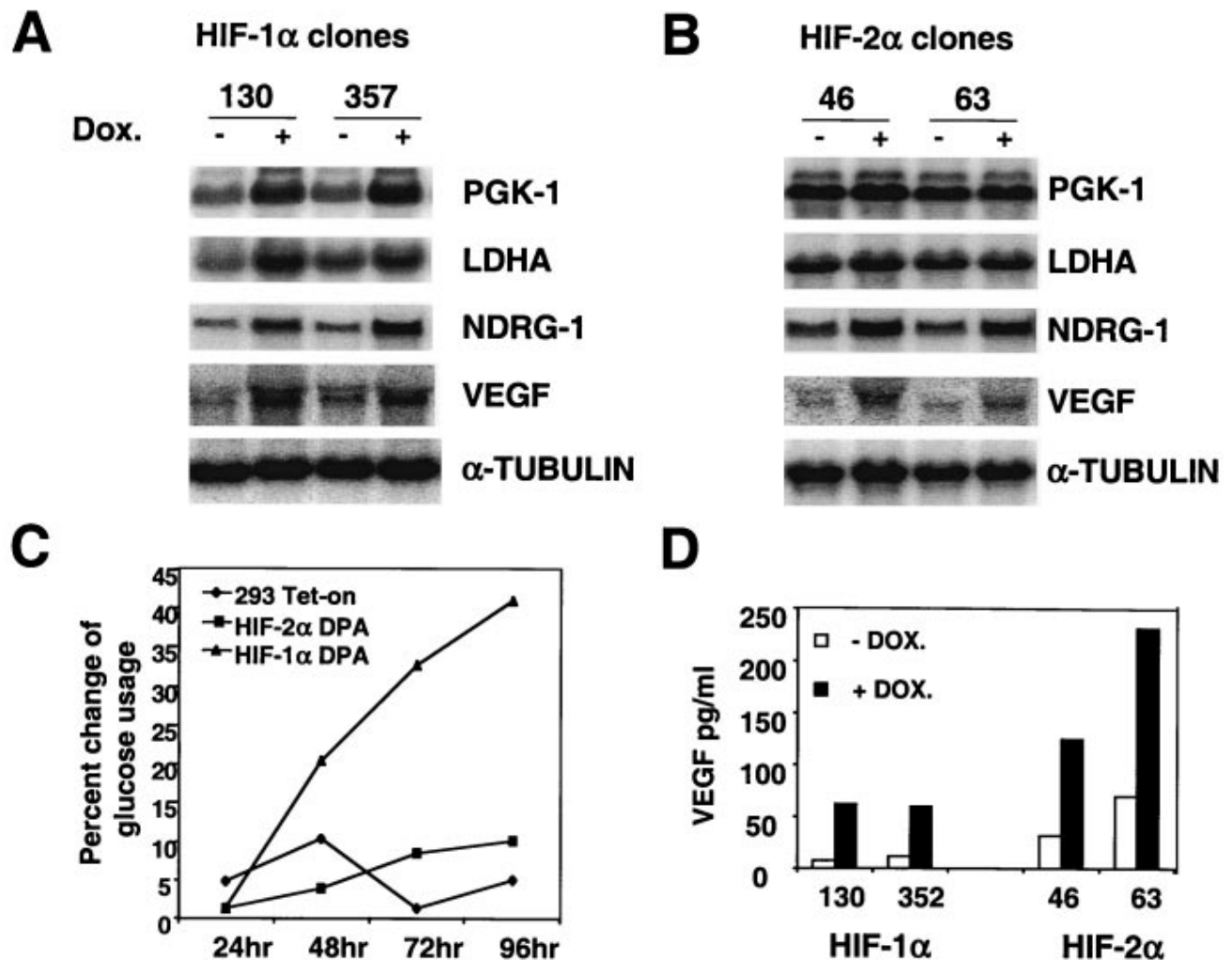


FIG. 7. HIF-1 and not HIF-2 stimulates genes encoding glycolytic enzymes in HEK293 TET-on cells. (A) Northern blot analysis of two glycolytic genes and two HIF-2-responsive genes in HEK293 TET-on HIF-1 DPA clones treated with 1g of doxycycline/ml for 20 h no doxycycline. HIF-1 increases the transcription of all four genes. (B) Northern blot analysis of two glycolytic genes and two HIF-2 - responsive genes in HEK293 TET-on HIF-2 DPA clones treated with 1g of doxycycline/ ml for 20 h. HIF-2 induced NDRG-1 and VEGF expression but not glycolytic gene expression. (C) Addition of doxycycline (1g/ml) increases the glucose usage of a HEK293 TET-on HIF-1DPA clone but not a HEK293 TET-on HIF-2DPA clone or HEK293 TET-on parental cells. The percent change of glucose usage was defined as [glucose (Dox) glucose (DOX)]/glucose (DOX). (D) Doxycycline treatment (1g/ml for 48 h) increases VEGF protein secretion in both HEK293 TET-on HIF-1DPA and HIF-2DPA clones.

HIF-1, but not HIF-2, promotes cellular glucose consumption

To further study the role of HIF-1 and HIF-2 in glucose metabolism, glucose consumption was determined in HEK293 TET-on HIF-1 DPA, HIF-2 DPA and

parental HEK293 TET-on cells by assaying glucose concentration in the culture media as a function of HIF-expression. Results are presented as percent change of glucose usage, defined as $[\text{glucose (no doxycycline)} - \text{glucose (with doxycycline)}] / \text{glucose (no doxycycline)}$. The addition of doxycycline significantly increased glucose consumption by HEK293 TET-on HIF-1 DPA clones after 48 h (figure 7C and data not shown). Although glycolytic gene expression was induced as early as 4 h post-treatment (data not shown), no significant glucose concentration change was observed until 48 h, indicating that there is a delay between glycolytic gene expression and increased glucose catabolism. However, doxycycline treatment of HEK293 TET-on HIF-2 DPA and parental HEK293 TET-on cells had no effect on glucose consumption, which is consistent with a Northern blot analysis showing that HIF-1 (but not HIF-2) increases glycolytic gene expression. On the other hand, doxycycline treatment enhanced VEGF protein secretion in both HEK293 TET-on HIF-1 DPA and HIF-2 DPA clones, correlating well with VEGF mRNA expression. These results indicate that both HIF-1 and HIF-2 regulate VEGF expression. Using the TET-on system in combination with normoxia functional HIF-1 and HIF-2, we clearly demonstrate a unique role for HIF-1 in glycolytic gene regulation and a common function for HIF-1 and HIF-2 in regulating a subset of hypoxia-responsive genes.

DISCUSSION

Functional studies of HIF-1 and HIF-2 are complicated by the fact that most cell lines express both HIF-1 and HIF-2 (see figure 3A) [42, 46]. *Hif-1* and *Hif-2*/ES cells would provide a perfect system to study HIF-2 target genes in *Hif-1*/ES cells or HIF-1 target genes in *Hif-2*/ES cells. Analysis of *Hif-1*/ES cells suggested that HIF-1, but not HIF-

2, plays a fundamental role in hypoxia responses [47- 49]. In agreement with this suggestion, analysis of *Hif-2* /ES cells indicated that HIF-2 is not involved in hypoxia in ES cells. In contrast to the ES cell studies, HIF-2 has been shown to be important in stimulating several hypoxia-inducible genes (including those for tyrosine hydroxylase and VEGF) during embryonic development [27, 50 and 51]. Different temporal and spatial expression patterns of HIF-1 and HIF-2 in the developing mouse embryo [52] could result in distinct phenotypes in mutant mice, even if the proteins regulate identical target genes. This possibility limits the usefulness of gene targeting experiments in studying HIF-1 and HIF-2 target genes. To avoid these issues, we began our studies of HIF-1 and HIF-2 in a defined cell type, 786-O cells. 786-O is one of the few cell lines in existence that is *VHL*-deficient with an accompanying genetic rescue of VHL [28, 29], allowing us to distinguish HIF-dependent regulation from HIF-independent hypoxic induction. In addition, 786-O cells express HIF-2 but not HIF-1 [30], enabling us to study the function of HIF-2 exclusively. Furthermore, 786-O WT-8 cells exhibit hypoxic induction of GLUT-1, VEGF, and PDGF-B, suggesting that the HIF-2 in 786-O cells is functional [29].

A genome-wide analysis of hypoxia-inducible genes with a DNA microarray and Northern blots demonstrated that a number of genes are induced by hypoxic treatment of 786-O WT- 8 cells (figure 2 and Table 1). Several novel hypoxia-inducible genes were identified, including those for ADRP, GRO-2, BIRC-3, and the Dmx-like 1 (DMXL-1) and nuclear factor IL-3-regulated (NFIL-3) genes. Interestingly, ADRP is frequently found at the surface of lipid droplets and appears to be important for lipid accumulation and lipid droplet formation [31, 32]. This suggests a possible link for its

function with the clear cell phenotype in *VHL*-defective renal carcinoma [53]. Several studies indicated that GRO-1, a homologue of GRO-2, acts synergistically with PDGF-B (hypoxia-stimulated in the 786-O cells) to regulate oligodendrocyte precursor proliferation in vitro and in vivo in the rat central nervous system [54, 55]. Moreover, the enhanced expression of GRO-1 by squamous cell carcinoma leads to accelerated tumor growth, angiogenesis, and metastasis in vivo [55]. The genes for ADRP and GRO-2 and other genes identified in our experiments (those for growth factors and cytokines IL-6 and IL-8 and angiogenic factors VEGF and ADM) appear to be important for cell growth and may play an important role in renal cell tumorigenesis.

TABLE 1. Genes induced in triplicate sets of hypoxic mRNAs as determined by DNA microarray assay (^a)

Gene	AC	Description	Avg fold induction
Confirmation			
ADRP	X97324	Adipose differentiation-related protein	2.1
Y			
NDRG-1	D87953	N-myc downstream regulated-1	2.3
Y			
APG5L	Y11588	APG5 (autophagy 5, <i>S. cerevisiae</i>)-like	1.8
ND			
DMXL-1	AJ005821	Dmx-like 1	2.4
Y			
GRO-2	M36820	GRO2 oncogene	2.3
ND			

CST	D88667	Cerebroside (3'-phosphoadenylylsulfate: galactosylceramide 3') sulfotransferase	1.6
ND			
NFIL-3	X64318	Nuclear factor, interleukin 3 regulated	1.5
ND			
AKAP-12	U81607	A kinase (PRKA) anchor protein (gravin) 12	1.6
ND			
POU5F-1	Z11898	POU domain, class 5, transcription factor 1	1.4
ND			
BIRC-3	U45878	Baculoviral IAP repeat-containing 3	1.8
ND			
AA142964	ESTs,	weakly similar to ORF YOR126c [<i>S. cerevisiae</i>]	1.4
ND			
KIAA0203	D86958	KIAA0203 gene product	1.9
ND			
PIK3CA	Z29090	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	1.9
Y			
DUSP-7	A1655015	Dual-specificity phosphatase 7	2.1
ND			
TPP-2	M73047	Tripeptidyl peptidase II	2.1
ND			
SLC16A4	U59185	Solute carrier family 16 (monocarboxylic acid transporters), member 4	3.1

ND			
KIAA0710	AB014610	KIAA0710 gene product	3.1
ND			
NDUFB6	A1302176	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 6 (17 kDa, B17)	2.4
ND			
GADD45A	M60974	Growth arrest and DNA-damage-inducible, alpha	1.5
Y			
GADD153	BC003637	Similar to DNA-damage-inducible transcript 3	1.7
Y			
IGFBP-3	M35878	Insulin-like growth factor binding protein 3	1.5
ND			
UNG	Y09008	Uracil-DNA glycosylase	1.7
ND			
VEGF	AF024710	Vascular endothelial growth factor	1.4
Y			
IL-6	X04430	Interleukin 6 (interferon, beta 2)	2.0
Y			
CA-12	AF037335	Carbonic anhydrase XII	2.2
Y			
FLG	M60502	Filaggrin	1.5
Y			
CCND-1	X59798	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	1.8

Y		
SLC2A1	K03195 Solute carrier family 2, member1	2.0
Y		
ADM	D14874 Adrenomedullin	2.3
Y		

(^a) The threshold indicative of an induced gene was set at 1.4-fold since Affymetrix DNA chips appeared to underestimate the differences between the values of the control and the experimental samples. AC, Gen-Bank accession number; Y, hypoxic induction confirmed by Northern blot assay; ND, not determined.

Analysis of the pattern of gene induction in *VHL*-deficient 786-O PRC-3 cells in combination with 786-O WT-8 (*VHL* functional) cells strongly suggests that increased expression of ADRP, NDRG-1, and VEGF is a result of HIF-2 activity. However, increased expression of GADD45A and CHOP is HIF-independent. Interestingly, glycolytic genes are not induced in 786-O cells, suggesting that HIF-2 is not able to target genes involved in glucose metabolism. In contrast, another renal clear cell carcinoma cell line expressing both HIF-1 and HIF-2 (RCC-4), exhibits HIF-dependent regulation of glycolytic gene expression (figure 2C), implying that HIF-1 is critical for glycolysis during O₂ deprivation. Furthermore, analysis of HIF-1 mRNA expression and glycolytic gene induction in multiple cell lines established a link between HIF-1 and glycolytic gene induction. Importantly, introduction of HIF-1 restores the glycolytic gene hypoxia response in otherwise genetically identical 786-O cells, demonstrating that HIF-1 exclusively targets glycolytic gene expression in this cell line

(figure 4). Although HIF-2 protein levels correlate well with HIF target gene expression in 786-O cells, there remains a slim possibility that hypoxic gene induction is regulated by some other HIF- subunit(s), such as HIF-3 [56] and/or unidentified HIF- . Alternatively, HIF-2 is functional, but its ability to regulate glycolytic genes is inhibited by HIF-3. The existence of multiple HIF- subunits and potential unidentified HIF- subunits compelled us to establish a system that would allow one to study the function of individual exogenous HIF- subunits.

The TET-regulated expression system is very useful for controlled gene expression. By using such a system, the function of HIF- subunits can be studied by directly comparing two genetically identical cells, one expressing HIF- by the addition of doxycycline and one lacking HIF- expression. This method of comparison avoids the clonal variability observed when the function of HIF- is assessed by comparing parental cells with HIF- transfectants. The TET-on system was chosen instead of the TET-off system in consideration of HIF- protein toxicity to cells. In addition, tTS, which blocks leaky expression, is only compatible with the TET-on system. The mutation of two critical proline residues in both HIF- subunits stabilized HIF-1 and HIF-2 under normoxia, allowing us to analyze the function of transfected HIF-only [45]. Clearly, HIF-1 activates glycolytic genes as well as other hypoxia-inducible genes identified in 786-O cells. However, as in 786-O cells, HIF-2 induced some hypoxia-inducible genes, but not the glycolytic genes, in HEK293 cells. Furthermore, glucose consumption was increased by the expression of HIF-1 and not HIF-2. On the other hand, VEGF protein secretion was enhanced by both factors. The data from the HEK293 TET-on system independently confirmed our results from 786-O cells

indicating that only HIF-1 regulates glycolytic gene expression during hypoxia. Thus, from studies of two cell lines (HEK293 and 786-O cells), we suggest that glycolytic genes are the unique targets of HIF-1. This finding is consistent with previous data indicating that HIF-1 is necessary for glycolytic capacity in multiple cells, such as granulocytes, monocytes/ macrophages [57], chondrocytes [58] and MEFs [59]. Therefore, HIF-2 does not target glycolytic genes in many cell types.

Besides protein stability, HIF transcriptional activity also appears to be regulated by O₂ levels (56, 57). Hydroxylation of a critical asparagine residue in the C-terminal transactivation domain is suppressed in hypoxic cells, promoting the interaction of HIF with the p300/CBP transcription coactivators and enhancing HIF transcriptional activity [60]. However, stabilized HIF-1 and HIF-2 appear functional in normoxic HEK293 cells as demonstrated by the transactivation of endogenous hypoxia-responsive gene expression (figure 7). No elevated induction of hypoxia-responsive gene expression was observed in the HEK293 TET-on HIF-DPA cells treated with doxycycline in combination with 1.5% O₂ (data not shown). It will be interesting to determine if the requirement for p300 is a cell type-specific event. Alternatively, if other alternative factor(s) can substitute for the function of p300? HIF-1 has been shown to be critical in regulating a variety of hypoxia-inducible genes in several systems. *Hif-1*/ES cells lost the hypoxic response of glycolytic genes GLUT-1 and VEGF (12, 42, and 83). This outcome is consistent with our results indicating that glycolytic genes are exclusively regulated by HIF-1 but inconsistent with our data stating that GLUT-1 and VEGF are also regulated by HIF-2. However, we found that HIF-2 is not functional in ES cells, although the stability and translocation of the HIF-2

protein into the nucleus are normally regulated in ES cells. Therefore, it is understandable that the loss of HIF-1 leads to a complete loss of HIF-dependent hypoxia responses in ES cells. Consistent with our results, HIF-2 has been shown to be dispensable for hypoxia responses with *Hif-2*/ES cells [6]. The critical role of HIF-1 in glycolysis has been documented in vivo as well as in other cells. With in situ hybridization, PGK-1 expression is totally eliminated in the margins of neural folds in *Hif-1* embryos [49]. Furthermore, no Pasteur Effect is observed in *Hif-1*/MEFs [59]. However, a study shows that HIF-2 is not functional in MEFs, due to its inability to translocate to the nucleus [61]. In contrast, our results clearly show that HIF-2 is functional in both 786-O cells and HEK293 cells but unable to regulate glycolytic gene expression.

ATP production is critical for the survival of any living organism. Shifting from oxidative phosphorylation to the O₂- independent glycolytic pathway is the most ancient cellular response to hypoxia, occurring even in *Caenorhabditis elegans* [62]. Interestingly, HIF-1 is conserved from *C. elegans* to humans and HIF-2 only exists in more complicated vertebrates such as chicken, quail, and mammals [63]. Involvement of HIF-1, and not HIF-2, in this ancient glycolytic pathway makes sense because HIF-1 is evolutionarily conserved and ubiquitously expressed. Although HIF-2 does not regulate glycolytic genes, it does stimulate GLUT-1 expression in 786-O and HEK293 TET-on cells. Increased glucose transport into cells may provide a survival advantage, since glucose is required for Akt mediated inhibition of cytochrome *c* release and apoptosis [14]. Akt has been shown to be activated by hypoxia [64]. Besides promoting glucose, uptake expression of the GLUT-1 protein itself also has been shown to be

critical for cell survival under unfavorable conditions [12]. Specifically, overexpression of GLUT-1 reduces hypoxia-induced apoptosis, possibly through down-regulation of c-Jun-NH2-terminal kinase activity [65].

Loss of HIF-1 renders normal cells incapable of supplying sufficient ATP through glycolysis, which explains why extensive endothelial and mesenchymal cell death was observed in *Hif-1*/embryos in the presence of normal amounts of VEGF [49]. This result demonstrates that HIF-1-dependent regulation of glycolytic genes plays an important role in normal embryonic development. However, increased glycolysis caused by HIF-1 may not be beneficial for tumor cells, which already exhibit elevated glycolysis due to other genetic mutations, since increased fermentation results in overproduction of lactic acid. Several reports indicate that p53 is only activated to promote apoptosis and cell cycle arrest in acidic environments [66]. Furthermore, the hypoxia-inducible proapoptotic gene bNIP-3 is also only active in acidic environments [5]. This unique function of HIF-1 in regulating the glycolytic pathway may partially explain why HIF-2, and not HIF-1 promotes tumorigenesis in 786-O renal clear cell carcinoma [67].

In the present work, one identified several HIF-2-inducible genes; however, these genes are also regulated by HIF-1. A number of in vitro functional studies with reporter assays indicate that several endothelial cell-specific genes (Tie-2 and Flk-1) are exclusively regulated by HIF-2 [68]. Currently, a search for genes uniquely regulated by HIF-2 is under way. One demonstrated that HIF-2 does not regulate endogenous glycolytic gene induction in 786-O and 293 cells. However, HIF-2 does activate the PGK-derived HRE-luciferase reporter (three tandem copies of HRE from PGK with

thymidine kinase promoter) (figure 5C), indicating that HIF-1 and HIF-2 may recognize similar core HREs and further suggesting that the structure of the whole promoter including the number and position of HREs may play an important role. HIF-2 activates some genes but not others in the same cells (786-O and 293), showing a clear promoter selectivity. However, the cis acting element important for the function of HIF-2 may not be the HRE. It will be interesting to see which cis-acting regulatory elements in hypoxia-inducible genes determine their distinct responsiveness to HIF-1 or HIF-2.

Conclusions

Several scientific studies reported that HIF-2 could affect and lead to aspects of different mechanisms relating to tumor growth. Our present work show that involvement of HIF-1, and not HIF-2, in this ancient glycolytic pathway makes sense because HIF-1 is evolutionarily conserved and ubiquitously expressed. The real effect in the hypoxic regulation of target gene expression and interaction with HIF-1 and HIF-2 in vivo is not well understood. More studies should be carried out in order to confirm this fact. In particular that there are another HIF family member, HIF-3, can also dimerize with ARNT and bind to HREs in vitro as it was reported by Gu et al. [69].

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