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Effect of Hypoxia Inducible Factor on Hypoxia Inducible Gene Expression and Glycolysis

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Aim: The effect of both HIF-1 and HIF-2 drastically affect hypoxia responses. Because the relative contributions to hypoxic gene induction and transcriptions are unknown; we aim to evaluate HIF2 target genes, and discuss the secondary gene activations and RNA expression transcripts.
Place and Duration of Study: This study was carried out in the Department of Biochemistry, Faculty of Science, Center of Innovation in Personalized Medicine, King Fahd Center for Medical Research, King Abdulaziz University, Jeddah, Saudi Arabia, in the year 2015.
Methodology: Both HIF-1α and HIF-2α have been implicated in hypoxia responses. However, till now, their relative contributions to hypoxic gene induction and transcriptions is unknown. Here in this study we evaluate HIF2α target genes, and discuss the secondary gene activations and RNA expression transcripts, specifically glycolytic genes and we believe such information can translate into further hypothesis generating studies that could result in oncology and drug discoveries.
Results: This study showed that, HFI1α stimulates glycolytic GE in both types of cells clearly showing for the HFI1α and HFI2α (or HFI1 and HFI2) 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

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understand the real effect of HFI2α in order to control/regulate different sides of digestive-tumors; including angiogenesis, proliferation and apoptosis, metastasis, metabolism and resistance to chemotherapy.

Conclusions: The study shows that HFI2α plays an essential and crucial role as regulator of the malignant phenotype. In the present study, one shows the importance role played by both HFI1 and 2 through tumor growth.

Keywords: HFI1α and HFI2α; tumor growth; malignant phenotype; regulated resistance.

1. INTRODUCTION

The hypoxia-inducible factor is complex-protein containing the well-known three proteins HIF1α (HFI1), HIF2α (HFI2). The amino-acid sequence of HIF 2α is compatible with HIF1 to about 48% [1-8]. In particular, HIF1 is a nitrogenous organic compound that consists of huge molecules of amino acids and it is a vital portion of all living cells. It constitutes an important part in the physiology of human responses to deficiencies of oxygen. HIF-1 is among the fundamental genes implicated in the metabolic mechanisms which keep the body functions in its normal and steady state; for example normal temperature, common fluid composition, average blood sugar, gas concentrations, blood pressure, etc. HIF-1 is also important for vital process such as defense against invading materials including virus, antigen or even bacteria/fungus. In addition, it fabricates defender materials (for example antibodies) against these overrun materials. In addition, HIF-1alpha is constitutionally expressed in most cells and that it rather than HIF-2alpha is able to cause an increase in transcription of genes which regulates glycolytic process and thus influence glucose utilization by tumors during a hypoxic state. In addition, the super family of transcription factors bHLH-PAS with HIF 2α and HIF 1β are the roots of HFI2α and HFI1β. We will denote them in this study as HFI1 and HFI2. HFI2α activates and relates transcription factors bHLH-PAS. It is, also, known as endothelial PAS domain protein-1 (EPAS1) [9-12]. PHDs introduce hydroxyl groups in order to yield two 2-proline residues: Pro 405 and 503 through HFI2α. This occurs by the aid of the cofactors O2, Fe and α-ketoglutarate. Thereafter, HFI2α joins to a conserved DNA sequence referred as HRE "the Hypoxia-response element"; then it Trans-activates a variety of HRGs "Hypoxia-responsive genes" [13-16]. Even though HFI2α shows stability at high

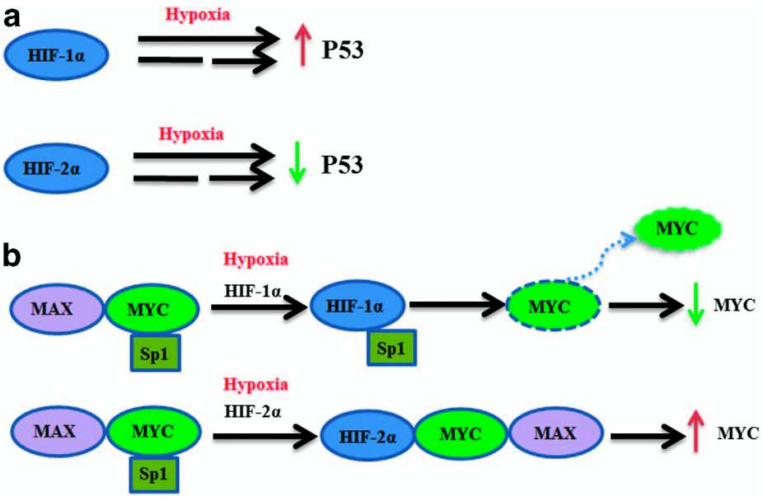

Fig. 1(a). Schematic illustration shows in full line hypoxia-direct and in dotted line hypoxia-indirect: The reverse effect of HIF-1α which up adjusts P53 activity while HIF-2α down adjusts P53 activity;

Fig. 1 (b). Schematic illustration shows HIF-1α and HIF-2α adjust in peculiar manner c-Myc reaction with its reproduction cofactors. HIF-1α reduces the assembly with MAX and SP1, and then reduces MYC action, while HIF-2α joins with MAX and SP1 complex, that way rising MYC action

O_2-pressure compared with HFI1α (*in vitro*), it has never been revealed under normxic conditions in various organs [17-19]. In addition, HFI2α is, also, induced with hypoxia [17-19]. Most cancer growth reveals Hypoxia as common event. Most of tumors and their corresponding metastases contain either HFI2α or HFI1α, or they can be present both [20-27]. The reverse effect of HIF-1α which up adjusts P53 activity while HIF-2α down adjusts P53 activity is schematized in Fig. 1 (a) and (b). In addition, bHLH-PAS is involved another protein in carrying out Hypoxia response [28-32]. In addition, from structure point of view, about 48% of the amino acid prints in HFI1α, HFI2α are similar with 83% similarities in their bHLH domains [13-16] and they have certain symmetric level in their O_2 dependent degradation domains. Moreover, the fifty amino acids groups situated "C" ends of HFI1α, HFI2α are strongly protected and show signs to improve the mechanism for O_2 regulation interactions with transcriptional-family p300 [33-38].

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 chose RCC-4 renal carcinoma RCC-4 (renal carcinoma cells) infected in our study because its subtype is well known. This presence of HepG2-cells, with HEK293, and Hep3B-cells helped culturing-processes in 1.5% oxygen in the presence of 1% fetal calf serum with 4.5 g of glucose/ml, 25 mM HEPES, for example for 21% oxygen for normoxia experiments or hypoxia-(Hypoxia). Human micro vascular endothelial cells (ENs) 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. [39], with the presence of TRIzol reagent, the Northern blot studies have been carried out conforming to standard protocols [40]. With the aid of using accession number from GenBank, different nucleotide (nt) have assessed the human DNA fragments. We carried out this with using retroviruses-"reverse transcription-PCR" and cloned into p-CRII-topo vector which represents standard and control material.

2.3 Hypoxia-responses: Arranging of Complementary DNA-RNA and DNA Microarray

We cultivated 786-O WT-8 cells in 21% oxygen for 24 hours with heterodimers of HIF-1 and HIF-2; then triplicate RNA-groups were arranged in order to initiate the DNA-microarray analysis. From total RNA with the Super- script Choice system (Gibco BRL Life Technologies) and T7-(dT)24 primers, one generates cDNA. In addition, biotin-labeled ribonucleotides were synthesized. In order to examine the fragmented complementary RNA for quality, we carried out two tests were tests were conducted in: The premier one, when examining an array; while the second test was using H-genome U95A arrays to mix atomic orbitals with 12,000 known mRNA. Gene-chip expression detailed examination software was used to get complete analysis of data [41,42]. Certain samples were experimentally investigated for average differentiation and total call. After that, the gene expression (GE) levels from two samples (Nox and Hypoxia) were subjected to comparative analysis for all three sets of data. In the presence of proteinase inhibitors, we used 200 M deferoxamine (DFX) to prepare nuclear extracts. Structure of Plasmid Full-length murine HFI1 or HFI2 single cDNA was generated by reverse transcription-PCR and by inserting into a pcDNA3 expression vector. This happened by using primers incorporated with restriction sites; as following:

For HFI1	5-ATAggatcc(*Bam*HI)ATGGAGGGCGCCGGCGGCGAGA AC-3 (sense)
	5 -CGCctcgag(*Xho*I)TCAGTTAACTTGATCCAAAGCTCT G-3(antisense).
For HFI2	5-CATggtacc(*Kpn*I)CGGC GACAATGACAGCTGACAAGGAG-3 (sense)
	5 -CATtctaga(*Xba*I)TCA GGTGGCCTGGTCCAGAGCTCTGAG-3 (antisense)

For HFI1 and HFI2: the start codon (ATG) and stop codon (TCA) is underlined letters.

By using "Pfu-PCR-based" technique, a 10-amino-acid c-myc tag was getting in at the C-end of the

two HIF-complementary DNAs. Similarly, the same "Pfu-PCR-based" technique was carried out to alter proline residues to alanines, one can write:

For HFI1	P402A, 5-GCTGCCGGCGACACCATCATCTCTC-3
	5-g gcAGCCAGCAGAGTGAGAGCATCAGGC-
For HFI2	P530A, 5-gcc-TAC ATCCCTATGGACGGCGAGG-3
	5-TGCCAAGGTCTCCAAATCCAG TTC-3

When using certain primer pairs, one amplified full-length-complementary DNA (HFI1 mycDPA-double prolines to alanines) or HFI2 mycDPA in pcDNA. In addition, it was installed into a TET response element (TRE) vector (pTRE2hyg; Clontech) by:

For HFI1	5 - ACCAacgcgt(MluI)CCGCCATGGAGGGCGCCGGCGGCGAGAAC-3
	5 - ACCAgcggccgc(NotI)TCACAGATCCTCTTCTGAGATGAG-3
For HFI2	5 - ACCAgcggccgc(NotI)TCACAGATCCTCTTCTGAGATGAG-3
	5 -ACCAacgcgt(MluI)CCGCCATGACAGCTGACAAGGAGAAAAAAAG G-3

HEK293 TET-on HIF-DPA clones were constructed by transfection of HEK293 TET-on cells (Clontech) with pTRE2hygHFI1 DPA or pTRE2hygHFI2 DPA in addition to pTet-tTS vector in the presence of 50 g of hygromycin/ml and 100 g of G418/ml.

One used Fugene 6 (Roche according to manufacturer's instructions) to carry out transfection of HEK293 cells with reporter and HIF- expression plasmid vectors. Antonioli et al [43] have reported the wild-type HRE-luciferase reporter as well as a mutant HRE-luciferase reporter. Using 200 ng of reporter, 200 ng of expression plasmid, and 100 ng of reference plasmid a murine sarcoma virus galactosidase, one could transfected 50 to 60% confluent HEK293 cells in a six-well plates. One transfected them for 40 hours transfection, and collect them in buffer (Promega). Then, depending on HIF- and -galactosidase activity, we accurately determined them for luciferase activity [44,45].

Glucose assay and VEGF enzyme-linked immunosorbent assay.

For analysis of glucose concentration and VEGF protein secretion assays, the same number of HEK293 TET-on HFI1 DPA or HEK293 TET-on HFI2 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 micro liter of the medium was collected for the glucose and VEGF assay at indicated times. The amount of glucose was measured using Sigma Diagnostics glucose reagents. VEGF quantization was performed with a QuantiKine human VEGF immunoassay kit (R&D Systems).

3. RESULTS AND DISCUSSION

Some authors revealed that HFI1 and HFI2 are involved in Hypoxia reactions [27] with unknown relative contributions to hypoxic gene. This is because the major part of O_2-regulated genes known in cells, which express HFI1 and HFI2. Iliopoulos et al. [46] selected a line 7860 WT-8 that researchers have extensively studied it, to evaluate HIF2. Moreover, the origin of cells of that line withdrawn from patients-under-treatment (PUT) with renal cell carcinoma and exhibited mutated VHL. From Fig. (2), one can notice that lanes 1 to 4 detect no HFI1 mRNA, Iliopoulos et al. [46] confirmed this data.

Fig. (2) shows that HFI1 and HFI2 are involved in Hypoxia Rs with unknown relative contributions to hypoxic gene. This is because the major part of O_2-regulated genes known in cells, which express HFI1 and HFI2, Lane 7860 WT-8 that researchers have extensively studied it, to evaluate HIF2.

From lanes 1 and 2 (Fig. 2), it is important to notice that no HFI1-protein was present even in the VHL-defective 786-O PRC-3 cells (786PCRs). These cells were acting with their VHL position: 786PCRs cultured at 1.5%-oxygen showed same standards of HFI2. Lanes 5 and 6 (Fig. 2) shows VHL-incomplete renal-carcinoma cell line, RCC-4, showed the two-proteins: HFI1 and HFI2. Lanes 7 and 8 (Fig. 2) show that soft and fixed transfection of pcDNA3-HA-tagged VHL (RCC-4T 3-14) lowers normoxic values of HFI1 and HFI2-protein, reinstituting Hypoxia inducibility. 786-O WT-8 cells, are cultured under Nox (21% oxygen-for 16 hours.) and used for making triplicate-samples of RNA- from 786-O WT-8 cells. Total RNA marked with Superscript

Choice System (Gibco BRL Life Technologies) and T7-(dT) 24 primers generate double strand cDNA. Afterwards, T7 DNA polymerase formed antisense biotin-labeled RNA. Using human genome-U95A arrays including about 12k known mRNA transcripts (Affymetrix), the RNA probe was chipped and hybridized. Moreover, novel Hypoxia- inducible genes for example 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 are induced genes. Nevertheless, platelet-derived growth factor, B chain (PDGF-B), is not on the human genome U95A arrays. In addition, one can found ADRP protein at the surface of lipid droplet that stimulates lipid droplet formation and lipid accumulation [47- 50].

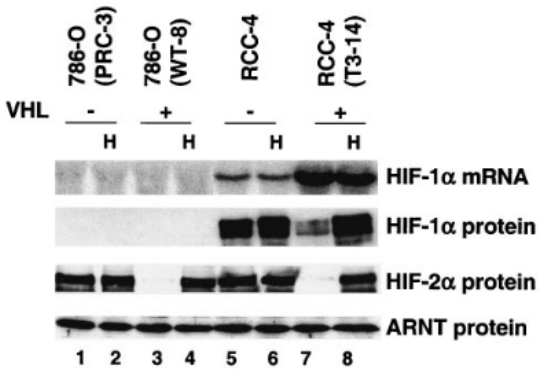

Fig. 2. HIF-2α is, only, expressed by 786-O cells and HIF-1α is not expressed

There is a phenomenon created when a sufficient amount of lipid droplets in some cells removed during sample preparation for microscopy may explains the over expression in VHL- defective renal clear cell carcinoma. DNA microarray assays identified some of this Hypoxia-IG and one used Northern blot analysis in triplicate RNA samples to more investigate and confirmed these Hypoxia-IG. Because the expression pattern of these genes was similar and identical in all three sets of RNA samples, therefore we showed only one set of Northern blotting data.

Predisposed to be HFI2 targets, containing a Hypoxia-inducible gene, the ADRP gene and three known Hypoxia-IG, NDRG-1 (2.0-fold), ADM (1.8-fold), and VEGF (1.8-fold) genes, Fig. 3 A lane 4 versus lane 3 illustrates there data. In order to unattached Hypoxia effects from HIF-dependent transcription stimulation, like for example Hypoxia-induced mRNA stability [51-54] or Hypoxia-induced AP-1 (4, 5) was characterized in otherwise genetically identical VHL-defective 786PCRs. Fig. 3A, lanes 1 and 2 versus lane 3 shows high standards of GE in 786-O PRC cells, independent of O_2 tension. In particular, the target GE (Fig. 3A) strongly related to HFI2 protein standards as shown in Fig. 2. Considering HFI2 stimulates the expression of these genes no other Hypoxia-stimulated transcription factors such as AP-1 and NF-B can do that. In addition, Fig. 2 lanes 5 to 8 shows that ADM (2.9-fold), VEGF (2.4-fold) ADRP (induction, 2.5-fold), and NDRG-1 (2.7-fold) are similarly activated by hypoxic treatment of RCC-4T 3-14. Nerveless, one cannot be sure if HIF-subunit affects the activation of RCC-4 gene or not? Fig. 3B lane 4 versus lane 3 shows that the expression of GADD153 and GADD45A (induction, 1.9-fold) are induced by less than average O_2. In addition, expression of CHOP and GADD45A become clear to be far dependent on HIF-/VHL pathway. This is because HFI2 protein standards not related to their expression HFI2 protein standards.

One can summarize the analysis, which was created by DNA microarray and Northern blot analysis in VHL-defective cells by Northern blot analysis in VHL-defective cells and DNA microarray and in cells with functional VHL showed that HFI2 is engaged in 786-O cells.

Moreover, several authors have reported that Hypoxia stimulates, expresses and activates genes involved in glucose metabolism [55-58]. Glycolytic-genes induce in Hypoxia-treated wild-type murine ES cells using similar DNA microarray analyses; however, even all the glycolytic genes are present on the human genome U95A DNA microarray; one did not observe glycolytic gene induction.

We used Northern blot analysis to test the expression of 13 glycolytic genes and we confirm that the DNA microarray data were present. Fig. 3C, lane 4 versus lane 3 illustrates that Hypoxia in 786-O WT-eight cells was not involved affecting on these genes. This figure illustrates the strong Hypoxia-responsive genes that related toPGK-1 and shows PGM-1 and PKM which have moderate response. In addition, Fig. 3C (lane 4 versus 3) shows no response to Hypoxia treatment in 786-O WT-8 cells and there are no responsive expression of the four-genes. Strong Hypoxia-treatment (0.1% O_2) did not affect this responsive expression as Fig. 3C (lane 5 versus

3) shows. Nevertheless, on the contradictory, Fig. 3A (lane 5 versus 3) shows for NDRG-1 (6.6-fold), VEGF (3.1-fold), ADM (3.7-fold), and ADRP (3.5-fold), exhibit high induction at 0.1% O2. One should note from Fig. 3C, lane 1 versus lane shows that, in 786-O WT-8 cells, the standards of the normoxic level due to glycolytic genes was able to be likened to that in VHL 786PCRs. This shows that HFI2 is without any impact on their expression. Fig. 3C, lanes 6 to 9 show that hypoxic induction of PKM (1.5-fold), LDHA (3.0 old), PGK (2.5-fold), and PGM-1 (1.6-fold) GE were observed from their origin RCC-4T 3-14 cells; this was carried out in parallel experiments. Fig. 3C, lanes 6-9 illustrate that HIF is a target of HIF, and consistent with their VHL status VHL status that affects the expression level of each gene.

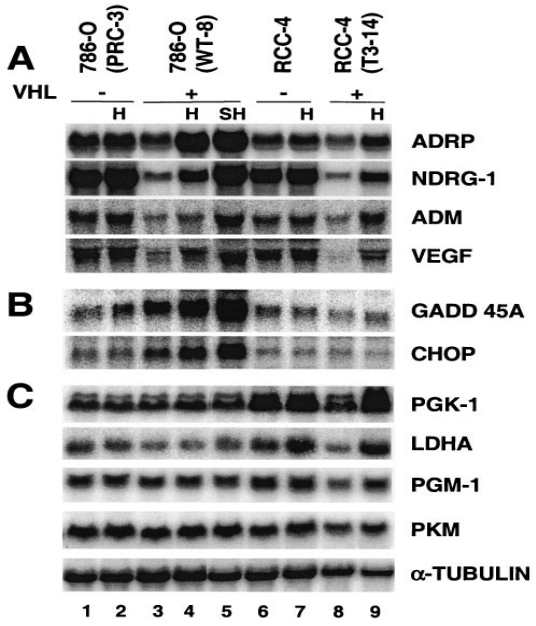

Fig. 3. Northern blot analyses and DNA microarray:
[a] Hypoxia (1.5% O_2 for 16 h)-[H] activates expression of VEGF, ADM, ADRP and NDRG-1 in cells-786-O (WT-8) and cells-RCC-4 (T3-14) [b] CHOP and GADD45A are Hypoxia-responsive genes but they are not correlated to HIF stimulation. [c] PKM mRNAs, LDHA,PGK-1, and PGM-1 that encodes four glycolytic enzymes

The standards of HIF-mRNA under Hypoxia were lowered with respect to that under Nox. This indicates that transcription of HFI1 and HFI2 is not raised by Hypoxia. Fig. 4B shows that "Hypoxia mimetic"-agents raised the standards of HFI1- and HFI2-proteins in all five-cell types. One found a good accord with HFI1 mRNA standards and all (but HMVEC-L) the 5-cell lines showed same levels of HFI1-protein. The much lower level of HFI1 protein in HMVEC-L cells (primary cells from lung) is consistent with several studies [59-60]. Taken in mind the mRNA standards, the two endothelial-cells showed elevated standards of HFI2 protein than 293. In the contradictory, Hep3B cells showed the highest standards of HFI2 protein. In addition, we noticed, in various cell lines, several standards of hypoxic gene induction. Moreover, HepG2 showed elevated standards of glycolytic gene induction [61]. It is worth noting that HepG2 is consistent with Hep3B cells (PGK, 4.5-fold; LDHA, 3.5-fold), PGK, 7.5-fold; LDHA, 4.4-fold and, feeble standards of induction were exhibited by HUVEC (PGK, 1.5-fold; LDHA, 1.8-fold), HEK293 cells (PGK, 2.5-fold; LDHA, 2.4-fold) and HMVEC-L (PGK, 1.5-fold; LDHA, 2.4-fold).

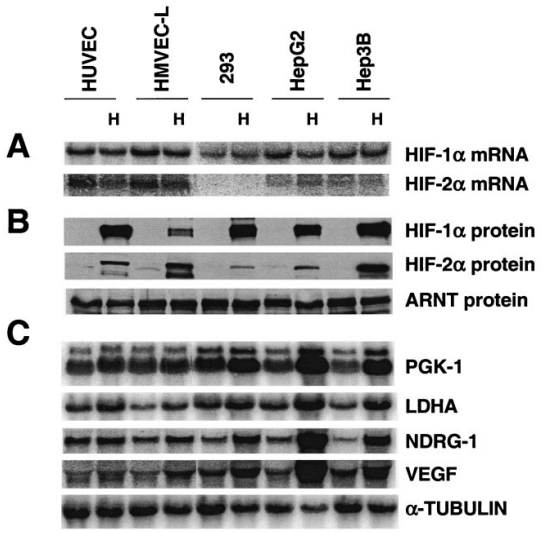

Fig. 4. Due to the effect of HIF1, glycolytic genes express hypoxic induction

Nevertheless, the poor value of induction did not an indication to specific glycolytic genes. Similarly, HFI2 IG: HMVEC-Ls (NDRG-1, 2.4-fold; VEGF, 2.4-fold), HUVECs (NDRG-1, 1.4-fold; VEGF, 1.6-fold), and HEK293s (NDRG-1, 2.8-fold; VEGF, 2.5-fold) showed poor levels.

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 HFI1 and HFI2. Importantly, all five HFI1-expressing cells exhibited glycolytic gene induction under low O_2 levels, consistent with a

role for HFI1 in regulating glycolytic enzyme expression (Fig. 4C).

4. CONCLUSIONS

We characterized various HIF2α, inducible genes,

1. These genes are adjusted by HIF1α
2. Several endothelial well-known Tie-2&Flk-1 show that HIF2α controls the processes,
3. HIF2α does not need to be controlled by endogenous glycolytic gene induction in 293cells&786O

CONSENT

Author has declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this paper and accompanying images.

ETHICAL APPROVAL

Author has hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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