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*Communication*

# Culture of cancer cells at physiological oxygen levels affects gene expression in a cell-type specific manner

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**Abstract:** Standard cell culture is routinely performed at supraphysiological oxygen concentrations (~18% $O_2$). Conversely, oxygen levels in most tissues range from 1%–6% (physioxia). Such hyperoxic conditions can alter reactive oxygen species (ROS) production, energy metabolism, mitochondrial network dynamics, and response to drugs and hormones. The aim of this project was to investigate the transcriptional response to different oxygen levels and whether it is similar across cell lines, or cell-line specific. Using RNA-seq, we performed differential gene expression and functional enrichment analyses in four human cancer cell lines, LNCaP, Huh-7, PC-3, and SH-SY5Y cultured at either 5% or 18% oxygen for 14 days. We found that oxygen levels affected transcript abundance of hundreds of genes, with the affected genes having little overlap between cell lines. Functional enrichment analysis also revealed different processes and pathways being affected in each cell line. Interestingly, we found that the top differentially expressed genes are involved in cancer biology. Further, we observed several hypoxia-inducible factor (HIF) targets upregulated at 5% oxygen, suggesting a role of HIF at physiological oxygen conditions. Finally, oxygen strongly induced transcription of mitochondrial genes in most cell lines, in a cell-type specific manner too. We conclude that cellular response to oxygen is widely cell-type specific, emphasizing the importance of maintaining physioxia in cell culture.

**Keywords:** oxygen; physioxia; hyperoxia; cell culture; transcriptomics; differential gene expression

## 1. Introduction

The goal of mammalian cell culture is typically to model cell function in vivo. However, while cells in most mammalian tissues are exposed to 1%–6% oxygen in vivo, cell culture is routinely performed in incubators that regulate $CO_2$ but not $O_2$, thus with $O_2$ equilibrating to ~18% $O_2$. Despite often referred to as 'normoxia', 18% $O_2$ is actually substantially hyperoxic relative to in vivo. An increasing amount of evidence has been showing that the hyperoxic conditions of cell culture affect multiple $O_2$-dependent processes, including ROS production [1], redox homeostasis [2], proliferation and differentiation [3], bioenergetics [4], mitochondrial network dynamics [4], and response to drugs [5] and hormones [6]. These effects of non-physiologically high $O_2$ levels can compromise the ability of cell culture models to recapitulate in vivo disease pathophysiology.

To gain a better understanding of how the hyperoxia of cell culture affects cancer cells, we used RNA-seq to analyze differential gene expression of four human cancer cell lines cultured for at least 14 days at either 5% $O_2$ (physioxia) or 18% $O_2$. The cell lines used in this project were LNCaP (prostate cancer), Huh-7 (hepatocarcinoma), PC-3 (prostate cancer), and SH-SY5Y (neuroblastoma). Using this approach, we asked whether transcriptomes of all four cell lines were sensitive to $O_2$ levels in this range, and whether the effects of $O_2$ were similar amongst cell lines, or cell-line specific. We found that the effects of $O_2$ on transcriptomes were substantial and highly cell-line specific.

## 2. Materials and Methods

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*2.1. Cell Culture*

LNCaP, SH-SY5Y, Huh-7, and PC-3 cell lines were purchased from ATCC (Manassas, VA, USA). Cells were cultured in 10-cm plates with Plasmax (Ximbio, London, UK) supplemented with 2.5% FBS and 1% penicillin/streptomycin (Sigma-Aldrich; St. Louis, MO, USA). All cell lines were incubated in a humidified 5% $CO_2$ incubator at either 5% or 18% $O_2$. Three replicates per each cell line were used in each condition. For the experimental groups kept at 5% $O_2$, Plasmax media was preincubated in the 5% $O_2$ incubator to allow for gas equilibration. Sub-culture was performed with 0.25% Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA) every 3 or 4 days, when cells reached ~80% confluence. Media was refreshed every 24 h. Cell culture was performed for 2 weeks. Cells were seeded at a density of $2x10^6$ cells/plate prior to RNA extraction.

*2.2. RNA Isolation*

Total RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, Toronto, ON, Canada) according to the manufacturer's instructions. RNA integrity was assessed using 1.5% agarose gel electrophoresis, while RNA concentration and purity were evaluated as A260/280 ratio using a Thermo Fisher Scientific Nanodrop spectrophotometer. RNA samples were snap frozen in liquid nitrogen and stored at –80°C until being sent to Novogene (Sacramento, CA, USA) for sequencing and analysis.

*2.3. Sequencing and Differential Gene Expression Analysis*

Quality check (QC), library preparation, and sequencing were performed by Novogene. Paired-end at 150 bp (PE150) high throughput Illumina sequencing was performed at a sequencing depth of 40 million reads per sample. Reads were aligned to the *Homo sapiens* reference genome (GRCh38) using Hisat2 v2.0.5 [7]. Gene expression levels were estimated by calculating FPKM (fragments per kilobase of transcript per million mapped sequence reads), which were further adjusted by edgeR program package [8] through one scaling normalized factor. Differential expression analysis was performed using the edgeR R package. A p-value<0.05 and $\log_2|FC|\geq 1$ were set as threshold for significantly differential expression.

*2.4. Functional Enrichment Analysis*

The list of DEGs was further reduced to genes with a Benjamini adjusted p-value ($p_{adj}$) < 0.1 and a FPKM≥1 in at least one of the experimental groups in order to produce a concise list of enrichment terms which reflect the most strongly affected genes. Functional enrichment analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [9]. Enriched Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and Reactome pathways were selected for functional annotation. A raw p-value and Benjamini adjusted p-value ($p_{adj}$) of 0.05 were applied for identifying the most statistically significant enriched annotation terms.

**3. Results**

*3.1. Oxygen levels in culture strongly modulated transcript abundance cell-line specifically*

The abundance of over a thousand transcripts were affected by oxygen in each cell line. In general, more DEGs showed higher expression at 5% $O_2$ than at 18% in all cell lines. In addition, there was substantial variation between the four cell lines in their sensitivities to $O_2$ (Figure 1A). For example, 2126 DEGs were identified in LNCaP cells, including 433 upregulated at 18% $O_2$ and 1693 at 5%. In contrast, SH-SY5Y was shown to be the least sensitive to $O_2$ among the cell lines, with only 386 transcripts upregulated at 18% $O_2$ and 848 at 5%. The full lists of DEGs for LNCaP, Huh-7, PC-3, and SH-SY5Y cells are available in Tables S1, S2, S3, and S4, respectively.

A remarkable result was the extremely limited overlap between cell lines in terms of the identities of the DEGs (Figure 1B). Only four genes were identified as being $O_2$-

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sensitive in all four cell lines. Even amongst the two prostate cancer cell lines, LNCaP and PC-3, where 2126 and 1461 were differentially expressed, respectively, only 192 were common to both cell lines. Similarly, of the 2099 transcripts affected by $O_2$ in Huh7 cells, 1638 were exclusively affected in this cell line. This indicates that $O_2$ effects on gene expression are highly specific to a given cell line. This in turn makes it difficult to predict how the non-physiological $O_2$ levels of standard cell culture are affecting cell biology in general terms.

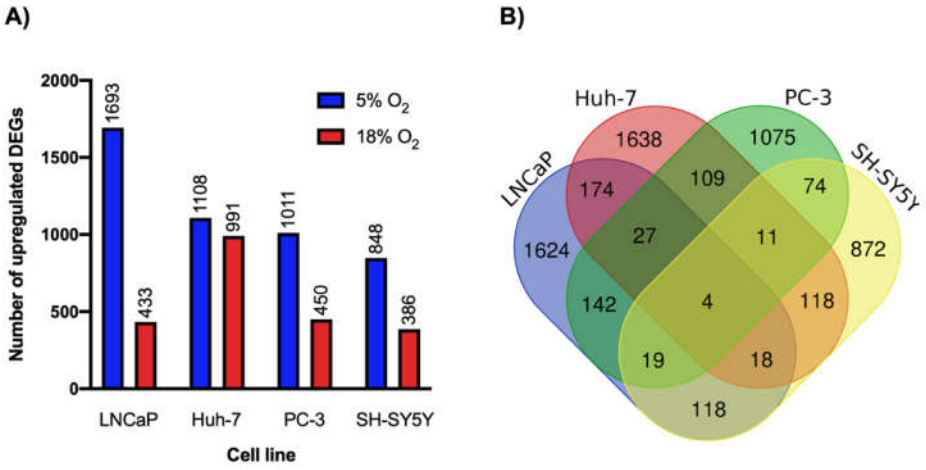


**Figure 1.** A) Number of differentially expressed genes (DEGs) upregulated at 5% and 18% $O_2$ in each cell line. B) Overlap of all DEGs affected by $O_2$ among the cell lines.

Functional enrichment analysis revealed that different biological processes and pathways were enriched by oxygen level in the four cell lines (Figure 2). For example, the most significantly affected pathway in LNCaP cells was TGF-β signaling ($p_{adj}$<0.05), which was found to be enriched at 18% $O_2$. In Huh-7 cells, pathways such as extracellular matrix (ECM) organization ($p_{adj}$<0.005) and drug metabolism by the cytochrome P450 (CYP450) enzymes ($p_{adj}$<0.05) were most strongly enriched at 18% $O_2$, while oxidative phosphorylation ($p_{adj}$<0.005) and oxidative stress-induced senescence ($p_{adj}$<0.05) were enriched at 5% $O_2$. Interestingly, in contrast to Huh-7 cells, both PC-3 and SH-SY5Y showed enrichment of annotation terms related to mitochondrial respiration and oxidative phosphorylation at 5% $O_2$ (see Figure 2). Signaling by interleukins ($p_{adj}$<0.05) and neurogenesis ($p_{adj}$<0.005) were among the processes enriched at 18% $O_2$ in SH-SY5Y cells. The full lists of functional annotation terms enriched by $O_2$ level in all cell lines are available in Tables S5–S12.

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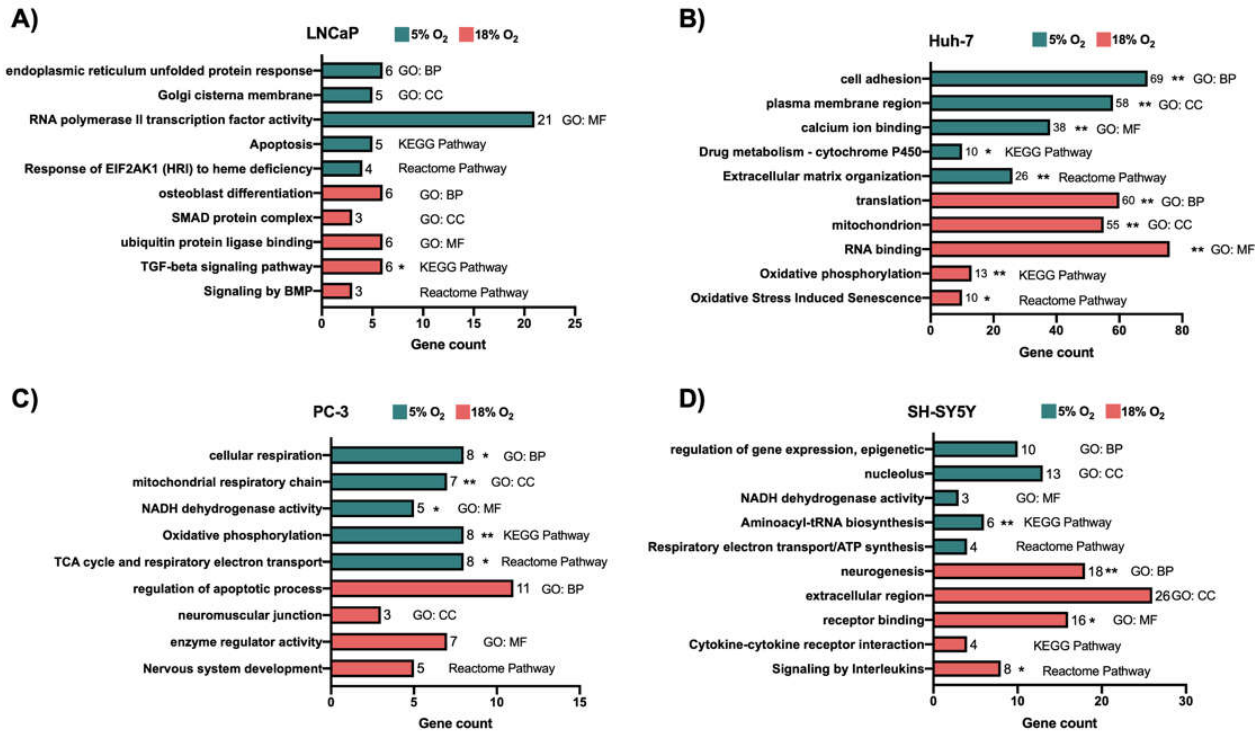


**Figure 2.** Selection of functional annotation terms enriched at 5% and 18% $O_2$ in **A)** LNCaP, **B)** Huh-7, **C)** PC-3, and **D)** SH-SY5Y cells. GO, gene ontology; CC, cellular component; MF, molecular function; KEGG, Kyoto encyclopedia of genes and genomes. (* $p_{adj}<0.05$, ** $p_{adj}<0.005$, otherwise $p<0.05$)

*3.2. The top differentially expressed genes have key roles in cancer cell biology*

By sorting the DEGs according to their adjusted p-value, we found that most of the genes highly affected by $O_2$ are implicated in cancer cell biology, including several with roles in cell proliferation, tumor progression, metastasis, invasion, and chemosensitivity to anticancer therapy. A selection of these genes is shown in Table 1. The complete list of top 10 DEGs in all cell lines at both $O_2$ conditions and their corresponding log2FC values is shown in Figure 3.

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**Table 1.** Selected DEGs with important roles in cancer biology

| Gene symbol | Gene name | Role in cancer biology | $Log_2FC$ at 18% vs. 5% $O_2$ † | Refs. |
|---|---|---|---|---|
| **LNCaP** | | | | |
| *ID1, ID3* ** | Inhibitor of DNA binding 1 and Inhibitor of DNA binding 3 | Transcription factor repressors; mediate metastasis, androgen resistance, and chemoresistance. | +2.50, +2.02 | [10] |
| *CHAC1* ** | ChaC glutathione specific gamma-glutamylcyclotransferase 1 | Degrades glutathione. Involved in ferroptosis; associated with increased chemosensitivity. | –2.81 | [11] |
| **Huh-7** | | | | |
| *S100A9* ** | S100 calcium binding protein A9 | TLR4 and RAGE ligand, promotes HCC progression through MAPK and NF-κB pathways. | +3.37 | [12] |
| *SLC3A2* ** (GLUT3) | Solute carrier family 2 member 3 | Selective glucose uniporter. Expression is correlated with HCC growth/invasion. | –5.74 | [13] |
| **PC-3** | | | | |
| *GREB1* | Growth regulating estrogen receptor binding 1 | Regulated by androgens, contributes to prostate cancer growth and antiandrogen resistance. | +7.03 | [14] |
| *ADAM33* * | ADAM metallopeptidase domain 33 | Methylation and upregulation observed in breast cancer. | –4.79 | [15] |
| **SH-SY5Y** | | | | |
| *MMP1* | Matrix metallopeptidase 1 | Upregulated in a wide variety of cancer types. | +1.90 | [16] |
| *CSAG2* ** (TRAG-3) | CSAG family member 2 | First isolated from taxol-resistant ovarian cancer cell line. Overexpressed in many cancer types, correlated with tumor progression. | –3.69 | [17] |

* padj<0.05, ** padj<0.005, otherwise p<0.05. †Positive value means gene is upregulated at 18% $O_2$ while negative value indicates upregulation at 5% $O_2$. Abbreviations: GLUT3, glucose transporter 3; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; RAGE, receptor for advanced glycation end-products; TLR4, toll-like receptor 4; TRAG-3, taxol-resistance associated gene 3.

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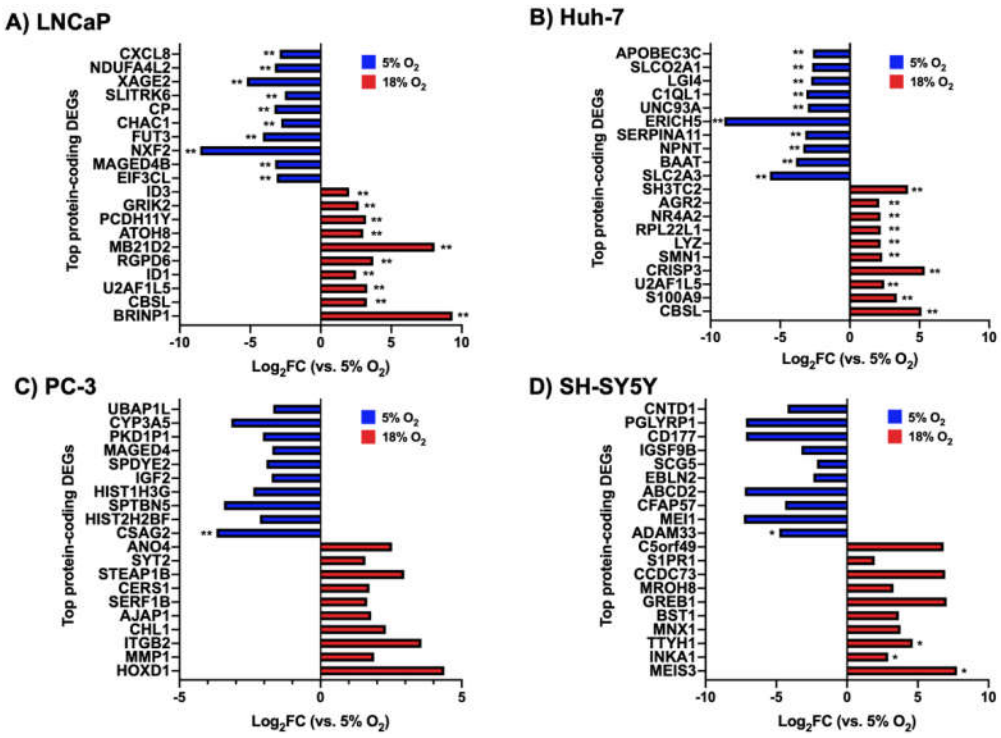


**Figure 3.** Top 10 differentially expressed genes in (**A**) LNCaP cells, (**B**) Huh-7 cells, **(C)** PC-3 cells, and **(D)** SH-SY5Y cells at 18% $O_2$ vs 5% $O_2$. (* $p_{adj}<0.05$, ** $p_{adj}<0.005$, otherwise $p<0.05$)

*3.2. Oxygen levels affected mtDNA-encoded transcript abundances in most cell lines*

Oxygen induced differential expression of mtDNA-encoded genes in most cell lines (Table 2). In Huh-7 cells, 11 mtDNA-encoded gene transcripts were affected by $O_2$, among which six are subunits of respiratory complexes I, IV, and V, while the rest are mitochondrial tRNAs. Interestingly, all of these were upregulated at 18% $O_2$. Eleven mtDNA-encoded genes were affected by $O_2$ in PC-3 cells and 10 in SH-SY5Y, however, all were upregulated at 5% $O_2$, in striking contrast with the observation in Huh-7 cells. Again, these DEGs encoded subunits of the respiratory chain or tRNAs. In contrast, only two mtDNA-encoded genes were affected in LNCaP. These results suggest that $O_2$ levels in cell culture affect the expression of mtDNA-encoded genes, but in a highly cell-type specific manner.

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**Table 2.** Mitochondrially-encoded genes differentially expressed at 5% or 18% $O_2$ in all cell lines

| Gene symbol | Gene name | Description/role | $Log_2FC$ at 18% $O_2$ vs 5% $O_2$ |
|---|---|---|---|
| **LNCaP** | | | |
| *MT-TL2* | Mitochondrially encoded tRNA leucine 2 (CUN) | Transfer RNA for leucine | –1.21 |
| *MT-TW* | Mitochondrially encoded tRNA trypto-phan | Transfer RNA for tryptophan | +1.82 |
| **Huh-7** | | | |
| *MT-ND1* | Mitochondrially encoded NADH:ubiqui-none oxidoreductase core subunit 1 | Complex I subunit | +1.36 |
| *MT-CO1* | Mitochondrially encoded cytochrome c oxidase I | Complex IV subunit | +1.07 |
| *MT-CO2* | Mitochondrially encoded cytochrome c oxidase II | Complex IV subunit | +1.19 |
| *MT-CO3* | Mitochondrially encoded cytochrome c oxidase III | Complex IV subunit | +1.13 |
| *MT-ATP6* | Mitochondrially encoded ATP synthase membrane subunit 6 | ATP synthase subunit | +1.07 |
| *MT-ATP8* | Mitochondrially encoded ATP synthase membrane subunit 8 | ATP synthase subunit | +1.03 |
| *MT-TY* * | Mitochondrially encoded tRNA tyrosine | Transfer RNA for tyrosine | +1.94 |
| *MT-TL1* | Mitochondrially encoded tRNA leucine 1 (UUA/G) | Transfer RNA for leucine | +1.44 |
| *MT-TV* | Mitochondrially encoded tRNA valine | Transfer RNA for valine | +2.07 |
| *MT-TW* | Mitochondrially encoded tRNA trypto-phan | Transfer RNA for tryptophan | +3.14 |
| *MT-TT* | Mitochondrially encoded tRNA threonine | Transfer RNA for threonine | +1.00 |
| **PC-3** | | | |
| *MT-ND2* | Mitochondrially encoded NADH:ubiqui-none oxidoreductase core subunit 2 | Complex I subunit | –1.08 |
| *MT-ND4* | Mitochondrially encoded NADH:ubiqui-none oxidoreductase core subunit 4 | Complex I subunit | –1.04 |
| *MT-ND4L* | Mitochondrially encoded NADH:ubiqui-none oxidoreductase core subunit 4L | Complex I subunit | –1.14 |
| *MT-ND5* | Mitochondrially encoded NADH:ubiqui-none oxidoreductase core subunit 5 | Complex I subunit | –1.01 |
| *MT-ND6* | Mitochondrially encoded NADH:ubiqui-none oxidoreductase core subunit 6 | Complex I subunit | –1.04 |
| *MT-CYB* | Mitochondrially encoded cytochrome b | Complex III subunit | –1.07 |
| *MT-CO2* | Mitochondrially encoded cytochrome c oxidase II | Complex IV subunit | –1.01 |
| *MT-ATP8* | Mitochondrially encoded ATP synthase membrane subunit 8 | ATP synthase subunit | –1.18 |
| *MT-TA* | Mitochondrially encoded tRNA alanine | Transfer RNA for alanine | –3.33 |
| *MT-TL1* | Mitochondrially encoded tRNA leucine 1 (UUA/G) | Transfer RNA for leucine | –1.07 |
| *MT-TM* | Mitochondrially encoded tRNA methio-nine | Transfer RNA for methionine | –2.29 |
| **SH-SY5Y** | | | |

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| | | | |
|---|---|---|---|
| *MT-ND3* | Mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 3 | Complex I subunit | –1.13 |
| *MT-ND5* | Mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 5 | Complex I subunit | –1.02 |
| *MT-ND6* | Mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 6 | Complex I subunit | –1.01 |
| *MT-ATP8* | Mitochondrially encoded ATP synthase membrane subunit 8 | ATP synthase subunit | –1.07 |
| *MT-TH* | Mitochondrially encoded tRNA histidine | Transfer RNA for histidine | –2.01 |
| *MT-TE* | Mitochondrially encoded tRNA glutamic acid | Transfer RNA for glutamate | –1.99 |
| *MT-TG* | Mitochondrially encoded tRNA glycine | Transfer RNA for glycine | –1.31 |
| *MT-TQ* | Mitochondrially encoded tRNA glutamine | Transfer RNA for glutamine | –6.27 |
| *MT-TT* | Mitochondrially encoded tRNA threonine | Transfer RNA for threonine | –1.38 |
| *MT-TS2* | Mitochondrially encoded tRNA serine 2 (AGU/C) | Transfer RNA for serine | –1.27 |

### *3.3. HIF-1/2 targets were found upregulated at 5% $O_2$ in LNCaP and Huh-7 cells*

Five percent $O_2$ is not hypoxic, and we have previously shown that, under the conditions used here, pericellular $O_2$ levels do not fall below 4% [18]. Nonetheless, previous reports have shown that HIF-1 activity is detectable at similar physiological $O_2$ levels [5,19]. Here we identified several HIF-1/2 gene targets upregulated at 5% $O_2$, particularly in LNCaP and Huh-7 cells (Table 3). In LNCaP, transcripts related to angiogenesis and vasodilation, such as *VEGFA* and *ADM*, were upregulated. Further, genes encoding enzymes involved in the metabolic reprograming of cells towards a glycolytic phenotype were upregulated in Huh-7 cells grown at 5% $O_2$. These genes include the glucose transporter *SLC2A3* (GLUT3), the glycolytic enzyme *ENO2* (enolase), and the gluconeogenic enzyme *PCK1* (phosphoenolpyruvate carboxykinase 1). Fewer HIF-1/2 targets were detected in PC-3 cells, and only one was found in SH-SY5Y, consistent with our initial observation that these two cell lines were less sensitive to $O_2$ than Huh-7 and LNCaP cells.

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**Table 3.** Differentially expressed HIF-1/2 targets upregulated at 5% $O_2$

| Gene symbol | Gene name | Role | Log2FC | Refs. † |
|---|---|---|---|---|
| **LNCaP** | | | | |
| *VEGFA* * | Vascular endothelial growth factor A | Promotes angiogenesis | 1.68 | [20] |
| *ADM* | Adrenomedullin | Vasodilator peptide | 1.62 | [21] |
| *CALCRL* | Calcitonin receptor like receptor | G protein-coupled receptor related to the calcitonin receptor; enables adrenomedullin binding activity | 1.63 | [22] |
| *ADORA2A* * | Adenosine A2a receptor | Activates adenylyl cyclase, inducing cAMP signaling | 2.19 | [23] |
| *NDUFA4L2* ** | NDUFA4, mitochondrial complex associated like 2 | Complex I subunit; shown to decrease $O_2$ consumption | 3.26 | [24] |
| *PLOD2* ** | Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 | Catalyzes the hydroxylation of lysyl residues in collagen-like peptides | 7.79 | [25] |
| *LOX* | Lysyl oxidase | Facilitates the crosslinking of collagens and elastin | 1.60 | [26] |
| *CP* ** | Ceruloplasmin | Involved in Cu transport | 3.28 | [27] |
| *TF* | Transferrin | Involved in Fe transport | 6.37 | [28] |
| *PMAIP1* | Phorbol-12-myristate-13-acetate-induced protein 1 | Pro-apoptotic protein | 1.18 | [29] |
| *ENG* | Endoglin | Auxiliary receptor for the TGF-β receptor complex | 1.43 | [30] |
| *STC2* | Stanniocalcin 2 | May have autocrine and paracrine functions; may be involved in $Ca^{2+}$ and phosphate transport and metabolism | 1.19 | [31] |
| *GPX8* | Glutathione peroxidase 8 | Catalyzes reduction of hydrogen and alkyl peroxides | 1.19 | [32] |
| *CXCL12* | C-X-C motif chemokine ligand 12 | Chemoattractant cytokine | 1.67 | [33] |
| **Huh-7** | | | | |
| *SLC2A3* ** | Solute carrier family 2 member 3 (GLUT3) | Selectively transports glucose into the cytosol | 5.74 | [34] |
| *HKDC1* | Hexokinase domain containing 1 | Novel member of the hexokinase family, involved in glucose metabolism | 1.51 | [35] |
| *PCK1* | Phosphoenolpyruvate carboxykinase 1 | Catalyzes conversion of PEP to oxaloacetate during gluconeogenesis | 1.49 | [36] |
| *ENO2* | Enolase 2 | Catalyzes conversion of 2-phosphoglycerate to PEP during glycolysis | 1.38 | [37] |
| *COX4I2* | Cytochrome c oxidase subunit 4I2 | Complex IV subunit; regulates efficiency of electron transport | 6.04 | [38] |
| *CP* ** | Ceruloplasmin | Involved in Cu transport | 1.84 | [27] |
| *LOXL2* * | Lysyl oxidase like 2 | Facilitates the crosslinking of collagens and elastin | 1.66 | [26] |
| *LOXL4* ** | Lysyl oxidase like 4 | Facilitates the crosslinking of collagens and elastin | 1.93 | [39] |
| *P4HA2* | Prolyl 4-hydroxylase subunit alpha 2 | Catalyzes the formation of 4-hydroxyproline during collagen synthesis | 1.14 | [25] |
| *PLOD2* | Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2 | Catalyzes the hydroxylation of lysyl residues in collagen-like peptides | 1.08 | [25] |
| *NPPB* ** | Natriuretic peptide B | Hormone that mediates natriuresis, diuresis, and vasodilation | 2.05 | [40] |
| *EPO* * | Erythropoietin | Promotes erythropoiesis | 1.45 | [41] |

| | | | | |
|---|---|---|---|---|
| *PDGFB* ** | Platelet derived growth factor subunit B | Potent mitogen and chemoattractant, promotes angiogenesis | 2.06 | [42] |
| *CXCL6* | C-X-C motif chemokine ligand 6 | Chemoattractant cytokine | 1.21 | [43] |
| *IGFBP1* ** | Insulin like growth factor binding protein 1 | Binds insulin-like growth factors, promotes migration and metabolism | 2.66 | [44] |
| *NDRG1* * | N-myc downstream regulated 1 | Involved in p53-mediated caspase activation and apoptosis | 1.53 | [45] |
| *EGLN3* | egl-9 family hypoxia inducible factor 3 (PHD-3) | Catalyzes hydroxylation of HIFs for subsequent degradation | 2.27 | [46] |
| *TFF2* | Trefoil factor 2 | May stabilize the mucus layer and affect healing of the epithelium | 1.19 | [47] |
| *HEY1* | Hes related family bHLH transcription factor with YRPW motif 1 | Transcriptional repressor; inhibits mitochondrial biogenesis in HCC | 1.06 | [48] |
| **PC-3** | | | | |
| *SLC2A9* | Solute carrier family 2 member 9 (GLUT9) | Transports glucose into the cytosol | 5.73 | [49] |
| *PDK1* | Pyruvate dehydrogenase kinase 1 | Phosphorylates and inhibits the pyruvate dehydrogenase complex | 1.03 | [50] |
| *CA9* | Carbonic anhydrase 9 | Catalyzes interconversion between $CO_2$ and $H_2O$ into carbonic acid | 1.98 | [51] |
| *TERT* | Telomerase reverse transcriptase | Mediates extension and replenishment of telomeres | 1.98 | [52] |
| *TH* | Tyrosine hydroxylase | Catalyzes the conversion of tyrosine to dopamine | 1.09 | [53] |
| *BNIP3* | BCL2 interacting protein 3 | Pro-apoptotic factor | 1.02 | [54] |
| **SH-SY5Y** | | | | |
| *IGF2* | Insulin like growth factor 2 | Promotes growth and proliferation | 1.74 | [55] |

* padj<0.05, ** padj<0.005, otherwise p<0.05. Abbreviations: cAMP, cyclic adenosine monophosphate; GLUT, glucose transporter; HCC, hepatocellular carcinoma; HIF; hypoxia-inducible factor; OMM, outer mitochondrial membrane; PEP, phosphoenolpyruvate; PHD-3, prolyl hydroxylase 3; ROS, reactive oxygen species; TGF-β, transforming growth factor-beta. †References identifying the gene as a HIF-1/2 target.

## 4. Discussion

The main goal of this study was to determine how the standard cell culture environment (18% $O_2$) impacts the cancer cell transcriptome compared to a more representative in vivo environment (5% $O_2$). We were particularly interested in the extent to which any effects of $O_2$ were shared amongst cell lines versus cell-line specific. Our results indicate broad transcriptional effects of $O_2$ between 5% and 18% that are highly cell-type specific. Even in LNCaP and PC-3, both prostate cancer cell lines, the overlap in $O_2$-dependent DEGs was just ~5%. These results are consistent with a previous study showing little overlap in the proteome of three diffuse large B-cell lymphoma cell lines cultured in the same two $O_2$ conditions [56]. Increased oxidative stress associated with higher $O_2$ levels affects a variety of pathways, including the p53 pathway and mitogen-activated protein kinase (MAPK) pathways (reviewed in [57]). Given that cancer cells have distinct mutations, including gene copy number differences, that affect these pathways, their differential transcriptional response to $O_2$ is perhaps not surprising. In any case, these observations highlight the importance of culturing cells at physioxia, since the effects of 18% $O_2$ are broad and may not be easy to predict.

Functional enrichment analysis revealed that biological processes and pathways relevant to the disease etiology of each cell type were altered by $O_2$ level. For example, prostate cancer commonly metastasizes to bone, forming primarily osteoblastic lesions. TGF-β and BMPs, released by prostate cancer cells, induce osteoblast differentiation, which in

turn release growth factors that stimulate the proliferation of prostate cancer cells [58]. The TGF-β signaling pathway was the annotation term most significantly enriched ($p_{adj}$<0.05) in LNCaP cells at 18% $O_2$. Signaling by bone morphogenic proteins (BMP) and osteoblast differentiation were also enriched (p<0.05). These interactions of key prostate cancer signaling pathways with $O_2$ level attest to the potential issues associated with a hyperoxic cell culture environment.

One of the main functions of hepatic cells is detoxification of xenobiotics through phase I (CYP450) and phase II enzymes. In Huh-7 cells, drug metabolism by the CYP450 was among the annotation terms enriched at 5% $O_2$ ($p_{adj}$<0.05), in accordance with a previous observation that CYP1A1, CYP1A2, and CYP2E1, along with a number of phase II enzymes, are upregulated in HepG2 cells cultured in physioxia, compared with cells at atmospheric $O_2$ [59]. Differential expression of phase I and II enzymes at different $O_2$ tensions leads to altered metabolism of drugs and toxins, which in turn results in altered biological responses to these compounds when tested in vitro.

We observed several HIF-1/2 targets upregulated at 5% $O_2$ in LNCaP and Huh-7 cells. Although HIF-1/2 are known as transcription factors in hypoxia, some studies have shown them to be active in the physiological $O_2$ range [5,19]. The fact that very few HIF-1/2 targets upregulated at 5% $O_2$ were observed in PC-3 and SH-SY5Y cells supports the notion that these cells are less sensitive to $O_2$ than LNCaP and Huh-7. Given the variety of mechanisms in tumorigenesis regulated by HIF (e.g., metabolism, migration, invasion, survival), complete loss of HIF-1/2 activity at 18% $O_2$ may compromise experiments focused on chemotherapeutic strategies.

Enrichment of functional annotation terms related to the mitochondria and/or mitochondrial processes (e.g., respiration) was observed in three of the four cell lines (Huh-7, PC-3 and SH-SY5Y), although the directions of the effects were inconsistent. While mitochondrial terms were enriched at 18% $O_2$ in Huh-7 cells, they were enriched at 5% $O_2$ in PC-3 and SH-SY5Y. The same general trend was apparent for mtDNA-encoded genes. Decreased expression of mtDNA-encoded genes in Huh-7 cells at 5% $O_2$ may be a direct consequence of decreased mitochondrial abundance. Indeed, mitochondrial biogenesis was one of the Reactome pathways enriched at 18% $O_2$ in Huh-7 cells ($p_{adj}$<0.05; see Table S8). Moradi et al. observed decreased mitochondrial footprint in Huh-7 cells grown at 5% $O_2$ compared to 18% $O_2$ [4]. Further, it has been shown that HEY1, a HIF-1 target, decreases mitochondrial biogenesis by repressing the expression of PTEN-induced kinase 1 (PINK1) in human hepatocellular carcinoma cells through a HIF-1 dependent mechanism. In accordance, our data shows *HEY1* expression increased 2.05-fold at 5% $O_2$ (p<0.05; see Table 3). Additionally, Zhang et al. reported that HIF-1 inhibits mitochondrial biogenesis in renal carcinoma cells by repressing PGC-1β [60]. Interestingly, expression of *PPARGC1B* was ~3 times lower in Huh-7 cells at 5% $O_2$ compared to 18% ($p_{adj}$<0.005; see Table S2). On the other hand, expression of all mtDNA-encoded genes affected by $O_2$ was higher in physioxia in both PC-3 and SH-SY5Y cells. Expression of genes related to mitochondrial biogenesis induced by HIF-1 was observed in the neuroblastoma cell line SK-N-AS when exposed to hypoxia, along with increased mtDNA copy numbers [61]. Moreover, mitochondrial abundance was higher in primary neurons grown at 2% and 5% $O_2$ compared to atmospheric $O_2$ [62]. No direct link between $O_2$ and regulation of mitochondrial biogenesis has been reported in prostate cancer. Future research should be directed to investigating the $O_2$-dependent mechanisms of mitochondrial gene expression and biogenesis in different cell types.

In conclusion, our results show that supraphysiological $O_2$ levels in cell culture significantly alter the global transcriptomes of cancer cell lines in highly cell line-specific ways. This makes it difficult to establish general rules regarding how non-physiological $O_2$ levels might affect experiments. In turn, this emphasizes the importance of cell culture in physioxia to increase the likelihood that results will translate to in vivo.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: DEG list LNCaP.xlsx; Table S2: DEG list Huh-7.xlsx; Table S3:

DEG list PC-3.xlsx; Table S4: DEG list SH-SY5Y.xlsx; Table S5 Functional Enrichment LNCaP 5% O2; Table S6 Functional Enrichment LNCaP 18% O2; Table S7 Functional Enrichment Huh-7 5% O2; Table S8 Functional Enrichment Huh-7 18% O2; Table S9 Functional Enrichment PC-3 5% O2; Table S10 Functional Enrichment PC-3 18% O2; Table S11 Functional Enrichment SH-SY5Y 5% O2; Table S12 Functional Enrichment SH-SY5Y 18% O2.

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## References

1. Maddalena, L.A.; Selim, S.M.; Fonseca, J.; Messner, H.; McGowan, S.; Stuart, J.A. Hydrogen Peroxide Production Is Affected by Oxygen Levels in Mammalian Cell Culture. *Biochemical and Biophysical Research Communications* **2017**, *493*, 246–251, doi:10.1016/j.bbrc.2017.09.037.
2. Ferguson, D.C.J.; Smerdon, G.R.; Harries, L.W.; Dodd, N.J.F.; Murphy, M.P.; Curnow, A.; Winyard, P.G. Altered Cellular Redox Homeostasis and Redox Responses under Standard Oxygen Cell Culture Conditions versus Physioxia. *Free Radical Biology and Medicine* **2018**, *126*, 322–333, doi:10.1016/j.freeradbiomed.2018.08.025.
3. Fehrer, C.; Brunauer, R.; Laschober, G.; Unterluggauer, H.; Reitinger, S.; Kloss, F.; Gülly, C.; Gaßner, R.; Lepperdinger, G. Reduced Oxygen Tension Attenuates Differentiation Capacity of Human Mesenchymal Stem Cells and Prolongs Their Lifespan. *Aging Cell* **2007**, *6*, 745–757, doi:10.1111/j.1474-9726.2007.00336.x.
4. Moradi, F.; Moffatt, C.; Stuart, J.A. The Effect of Oxygen and Micronutrient Composition of Cell Growth Media on Cancer Cell Bioenergetics and Mitochondrial Networks. *Biomolecules* **2021**, *11*, 1177, doi:10.3390/biom11081177.
5. Yan, H.M.; Ramachandran, A.; Bajt, M.L.; Lemasters, J.J.; Jaeschke, H. The Oxygen Tension Modulates Acetaminophen-Induced Mitochondrial Oxidant Stress and Cell Injury in Cultured Hepatocytes. *Toxicological Sciences* **2010**, *117*, 515–523, doi:10.1093/toxsci/kfq208.
6. Moradi, F.; Fiocchetti, M.; Marino, M.; Moffatt, C.; Stuart, J.A. Media Composition and O2 Levels Determine Effects of 17b-Estradiol and Selective Estrogen Receptor Modulators on Mitochondrial Bioenergetics and Cellular Reactive Oxygen Species. *American Journal of Physiology - Cell Physiology* **2021**, *321*, C72–C81, doi:10.1152/ajpcell.00080.2021.
7. Kim, D.; Paggi, J.M.; Park, C.; Bennett, C.; Salzberg, S.L. Graph-Based Genome Alignment and Genotyping with HISAT2 and HISAT-Genotype. *Nature Biotechnology* **2019**, *37*, 907–915, doi:10.1038/s41587-019-0201-4.
8. Robinson, M.D.; McCarthy, D.J.; Smyth, G.K. EdgeR: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data. *Bioinformatics* **2009**, *26*, 139–140, doi:10.1093/bioinformatics/btp616.
9. Huang, D.W.; Sherman, B.T.; Lempicki, R.A. Systematic and Integrative Analysis of Large Gene Lists Using DAVID Bioinformatics Resources. *Nature Protocols* **2009**, *4*, 44–57.
10. Asirvatham, A.J.; Carey, J.P.W.; Chaudhary, J. Id1-, Id2-, and Id3-Regulated Gene Expression in E2A Positive or Negative Prostate Cancer Cells. *Prostate* **2007**, *67*, 1411–1420, doi:10.1002/pros.20633.

11. He, S.; Zhang, M.; Ye, Y.; Zhuang, J.; Ma, X.; Song, Y.; Xia, W. ChaC Glutathione Specific Γ-glutamylcyclotransferase 1 Inhibits Cell Viability and Increases the Sensitivity of Prostate Cancer Cells to Docetaxel by Inducing Endoplasmic Reticulum Stress and Ferroptosis. *Experimental and Therapeutic Medicine* **2021**, *22*, 997, doi:10.3892/etm.2021.10429.
12. Wu, R.; Duan, L.; Cui, F.; Cao, J.; Xiang, Y.; Tang, Y.; Zhou, L. S100A9 Promotes Human Hepatocellular Carcinoma Cell Growth and Invasion through RAGE-Mediated ERK1/2 and P38 MAPK Pathways. *Experimental Cell Research* **2015**, *334*, 228–238, doi:10.1016/j.yexcr.2015.04.008.
13. Gao, H.; Hao, Y.; Zhou, X.; Li, H.; Liu, F.; Zhu, H.; Song, X.; Niu, Z.; Ni, Q.; Chen, M.S.; et al. Prognostic Value of Glucose Transporter 3 Expression in Hepatocellular Carcinoma. *Oncology Letters* **2020**, *19*, 691–699, doi:10.3892/ol.2019.11191.
14. Rae, J.M.; Johnson, M.D.; Cordero, K.E.; Scheys, J.O.; Larios, J.M.; Gottardis, M.M.; Pienta, K.J.; Lippman, M.E. GREB1 Is a Novel Androgen-Regulated Gene Required for Prostate Cancer Growth. *Prostate* **2006**, *66*, 886–894, doi:10.1002/pros.20403.
15. Yang, P.J.; Hou, M.F.; Tsai, E.M.; Liang, S.S.; Chiu, C.C.; Ou-Yang, F.; Kan, J.Y.; Peng, C.Y.; Wang, T. nai Breast Cancer Is Associated with Methylation and Expression of the a Disintegrin and Metalloproteinase Domain 33 (ADAM33) Gene Affected by Endocrine-Disrupting Chemicals. *Oncology Reports* **2018**, *40*, 2766–2777, doi:10.3892/or.2018.6675.
16. Gobin, E.; Bagwell, K.; Wagner, J.; Mysona, D.; Sandirasegarane, S.; Smith, N.; Bai, S.; Sharma, A.; Schleifer, R.; She, J.X. A Pan-Cancer Perspective of Matrix Metalloproteases (MMP) Gene Expression Profile and Their Diagnostic/Prognostic Potential. *BMC Cancer* **2019**, *19*, 581, doi:10.1186/s12885-019-5768-0.
17. Duan, Z.; Feller, A.J.; Toh, H.C.; Makastorsis, T.; Seiden, M. v TRAG-3, a Novel Gene, Isolated from a Taxol-Resistant Ovarian Carcinoma Cell Line k. *Gene* **1999**, *229*, 75–81.
18. Gardner, G.; Moradi, F.; Moffatt, C.; Cliche, M.; Garlisi, B.; Gratton, J.; Mehmood, F.; Stuart, J.A. Rapid Nutrient Depletion to below the Physiological Range by Cancer Cells Cultured in Plasmax. *American Journal of Physiology-Cell Physiology* **2022**, doi:10.1152/ajpcell.00403.2021.
19. Bracken, C.P.; Fedele, A.O.; Linke, S.; Balrak, W.; Lisy, K.; Whitelaw, M.L.; Peet, D.J. Cell-Specific Regulation of Hypoxia-Inducible Factor (HIF)-1α and HIF-2α Stabilization and Transactivation in a Graded Oxygen Environment. *Journal of Biological Chemistry* **2006**, *281*, 22575–22585, doi:10.1074/jbc.M600288200.
20. Jubb, A.M.; Pham, T.Q.; Hanby, A.M.; Frantz, G.D.; Peale, F. v.; Wu, T.D.; Koeppen, H.W.; Hillan, K.J. Expression of Vascular Endothelial Growth Factor, Hypoxia Inducible Factor 1α, and Carbonic Anhydrase IX in Human Tumours. *Journal of Clinical Pathology* **2004**, *57*, 504–512, doi:10.1136/jcp.2003.012963.
21. Garayoa, M.; Martínez, A.; Lee, S.; Pío, R.N.; An, W.G.; Neckers, L.; Trepel, J.; Montuenga, L.M.; Ryan, H.; Johnson, R.; et al. Hypoxia-Inducible Factor-1 (HIF-1) Up-Regulates Adrenomedullin Expression in Human Tumor Cell Lines during Oxygen Deprivation: A Possible Promotion Mechanism of Carcinogenesis. *Molecular Endocrinology* **2000**, *14*, 848–862.
22. Nikitenko, L.L.; Smith, D.M.; Bicknell, R.; Rees, M.C.P. Transcriptional Regulation of the CRLR Gene in Human Microvascular Endothelial Cells by Hypoxia. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2003**, *17*, 1499–1501, doi:10.1096/fj.02-0993fje.
23. Ahmad, A.; Ahmad, S.; Glover, L.; Miller, S.M.; Shannon, J.M.; Guo, X.; Franklin, W.A.; Bridges, J.P.; Schaack, J.B.; Colgan, S.P.; et al. Adenosine A 2A Receptor Is a Unique Angiogenic Target of HIF-2 in Pulmonary Endothelial Cells. *Proc Natl Acad Sci U S A* **2009**, *106*, 10684–10689.
24. Tello, D.; Balsa, E.; Acosta-Iborra, B.; Fuertes-Yebra, E.; Elorza, A.; Ordóñez, Á.; Corral-Escariz, M.; Soro, I.; López-Bernardo, E.; Perales-Clemente, E.; et al. Induction of the Mitochondrial NDUFA4L2 Protein by HIF-1α Decreases Oxygen Consumption by Inhibiting Complex i Activity. *Cell Metabolism* **2011**, *14*, 768–779, doi:10.1016/j.cmet.2011.10.008.
25. Gilkes, D.M.; Bajpai, S.; Chaturvedi, P.; Wirtz, D.; Semenza, G.L. Hypoxia-Inducible F0actor 1 (HIF-1) Promotes Extracellular Matrix Remodeling under Hypoxic Conditions by Inducing P4HA1, P4HA2, and PLOD2 Expression in Fibroblasts. *Journal of Biological Chemistry* **2013**, *288*, 10819–10829, doi:10.1074/jbc.M112.442939.

26. Schietke, R.; Warnecke, C.; Wacker, I.; Schödel, J.; Mole, D.R.; Campean, V.; Amann, K.; Goppelt-Struebe, M.; Behrens, J.; Eckardt, K.U.; et al. The Lysyl Oxidases LOX and LOXL2 Are Necessary and Sufficient to Repress E-Cadherin in Hypoxia: Insights into Cellular Transformation Processes Mediated by HIF-1. *Journal of Biological Chemistry* **2010**, *285*, 6658–6669, doi:10.1074/jbc.M109.042424.
27. Mukhopadhyay, C.K.; Mazumder, B.; Fox, P.L. Role of Hypoxia-Inducible Factor-1 in Transcriptional Activation of Ceruloplasmin by Iron Deficiency. *Journal of Biological Chemistry* **2000**, *275*, 21048–21054, doi:10.1074/jbc.M000636200.
28. Rolfs, A.; Kvietikova, I.; Gassmann, M.; Wenger, R.H. Oxygen-Regulated Transferrin Expression Is Mediated by Hypoxia-Inducible Factor-1. *Journal of Biological Chemistry* **1997**, *272*, 20055–20062, doi:10.1074/jbc.272.32.20055.
29. Kim, J.Y.; Ahn, H.J.; Ryu, J.H.; Suk, K.; Park, J.H. BH3-Only Protein Noxa Is a Mediator of Hypoxic Cell Death Induced by Hypoxia-Inducible Factor 1α. *Journal of Experimental Medicine* **2004**, *199*, 113–123, doi:10.1084/jem.20030613.
30. Sánchez-Elsner, T.; Botella, L.M.; Velasco, B.; Langa, C.; Bernabéu, C. Endoglin Expression Is Regulated by Transcriptional Cooperation between the Hypoxia and Transforming Growth Factor-β Pathways. *Journal of Biological Chemistry* **2002**, *277*, 43799–43808, doi:10.1074/jbc.M207160200.
31. Law, A.Y.S.; Wong, C.K.C. Stanniocalcin-2 Is a HIF-1 Target Gene That Promotes Cell Proliferation in Hypoxia. *Experimental Cell Research* **2010**, *316*, 466–476, doi:10.1016/j.yexcr.2009.09.018.
32. Bosello-Travain, V.; Forman, H.J.; Roveri, A.; Toppo, S.; Ursini, F.; Venerando, R.; Warnecke, C.; Zaccarin, M.; Maiorino, M. Glutathione Peroxidase 8 Is Transcriptionally Regulated by HIFα and Modulates Growth Factor Signaling in HeLa Cells. *Free Radical Biology and Medicine* **2015**, *81*, 58–68, doi:10.1016/j.freeradbiomed.2014.12.020.
33. Hitchon, C.; Wong, K.; Ma, G.; Reed, J.; Lyttle, D.; El-Gabalawy, H. Hypoxia-Induced Production of Stromal Cell-Derived Factor 1 (CXCL12) and Vascular Endothelial Growth Factor by Synovial Fibroblasts. *Arthritis and Rheumatism* **2002**, *46*, 2587–2597, doi:10.1002/art.10520.
34. O'Rourke, J.F.; Pugh, C.W.; Bartlett, S.M.; Ratcliffe, P.J. Identification of Hypoxically Inducible MRNAs in HeLa Cells Using Differential-Display PCR Role of Hypoxia-Inducible Factor-1. *Eur. J. Biochem* **1996**, *241*, 403–410.
35. Askoxylakis, V.; Millonig, G.; Wirkner, U.; Schwager, C.; Rana, S.; Altmann, A.; Haberkorn, U.; Debus, J.; Mueller, S.; Huber, P.E. Investigation of Tumor Hypoxia Using a Two-Enzyme System for in Vitro Generation of Oxygen Deficiency. *Radiation Oncology* **2011**, *6*, doi:10.1186/1748-717X-6-35.
36. Owczarek, A.; Gieczewska, K.; Jarzyna, R.; Jagielski, A.K.; Kiersztan, A.; Gruza, A.; Winiarska, K. Hypoxia Increases the Rate of Renal Gluconeogenesis via Hypoxia-Inducible Factor-1-Dependent Activation of Phosphoenolpyruvate Carboxykinase Expression. *Biochimie* **2020**, *171–172*, 31–37, doi:10.1016/j.biochi.2020.02.002.
37. Leiherer, A.; Geiger, K.; Muendlein, A.; Drexel, H. Hypoxia Induces a HIF-1α Dependent Signaling Cascade to Make a Complex Metabolic Switch in SGBS-Adipocytes. *Molecular and Cellular Endocrinology* **2014**, *383*, 21–31, doi:10.1016/j.mce.2013.11.009.
38. Fukuda, R.; Zhang, H.; Kim, J. whan; Shimoda, L.; Dang, C. v.; Semenza, G.L.L. HIF-1 Regulates Cytochrome Oxidase Subunits to Optimize Efficiency of Respiration in Hypoxic Cells. *Cell* **2007**, *129*, 111–122, doi:10.1016/j.cell.2007.01.047.
39. Wong, C.C.L.; Gilkes, D.M.; Zhang, H.; Chen, J.; Wei, H.; Chaturvedi, P.; Fraley, S.I.; Wong, C.M.; Khoo, U.S.; Ng, I.O.L.; et al. Hypoxia-Inducible Factor 1 Is a Master Regulator of Breast Cancer Metastatic Niche Formation. *Proc Natl Acad Sci U S A* **2011**, *108*, 16369–16374, doi:10.1073/pnas.1113483108.
40. Weidemann, A.; Klanke, B.; Wagner, M.; Volk, T.; Willam, C.; Wiesener, M.S.; Eckardt, K.U.; Warnecke, C. Hypoxia, via Stabilization of the Hypoxia-Inducible Factor HIF-1α, Is a Direct and Sufficient Stimulus for Brain-Type Natriuretic Peptide Induction. *Biochemical Journal* **2008**, *409*, 233–242, doi:10.1042/BJ20070629.
41. Grimm, C.; Wenzel, A.; Groszer, M.; Mayser, H.; Seeliger, M.; Samardzija, M.; Bauer, C.; Gassmann, M.; Remé, C.E. HIF-1-Induced Erythropoietin in the Hypoxic Retina Protects against Light-Induced Retinal Degradation. *Nature Medicine* **2002**, *8*, 718–724.

42. Schito, L.; Rey, S.; Tafani, M.; Zhang, H.; Wong, C.C.L.; Russo, A.; Russo, M.A.; Semenza, G.L. Hypoxia-Inducible Factor 1-Dependent Expression of Platelet-Derived Growth Factor B Promotes Lymphatic Metastasis of Hypoxic Breast Cancer Cells. *Proc Natl Acad Sci U S A* **2012**, *109*, doi:10.1073/pnas.1214019109.
43. Tian, H.; Huang, P.; Zhao, Z.; Tang, W.; Xia, J. HIF-1α Plays a Role in the Chemotactic Migration of Hepatocarcinoma Cells through the Modulation of CXCL6 Expression. *Cellular Physiology and Biochemistry* **2014**, *34*, 1536–1546, doi:10.1159/000366357.
44. Tazuke, S.I.; Mazure, N.M.; Sugawara, J.; Carland, G.; Faessen, G.H.; Suen, L.-F.; Irwin, J.C.; Powell, D.R.; Giaccia, A.J.; Giudice, L.C. Hypoxia Stimulates Insulin-like Growth Factor Binding Protein 1 (IGFBP-1) Gene Expression in HepG2 Cells: A Possible Model for IGFBP-1 Expression in Fetal Hypoxia. *Proc Natl Acad Sci U S A* **1998**, *95*, 10188–10193.
45. Guo, D.D.; Xie, K.F.; Luo, X.J. Hypoxia-Induced Elevated NDRG1 Mediates Apoptosis through Reprograming Mitochondrial Fission in HCC. *Gene* **2020**, *741*, doi:10.1016/j.gene.2020.144552.
46. Pescador, N.; Cuevas, Y.; Naranjo, S.; Alcaide, M.; Villar, D.; Landázuri, M.O.; del Peso, L. Identification of a Functional Hypoxia-Responsive Element That Regulates the Expression of the Egl Nine Homologue 3 (Egln3/Phd3) Gene. *Biochemical Journal* **2005**, *390*, 189–197, doi:10.1042/BJ20042121.
47. Hernández, C.; Santamatilde, E.; McCreath, K.J.; Cervera, A.M.; Díez, I.; Ortiz-Masiá, D.; Martínez, N.; Calatayud, S.; Esplugues, J. v.; Barrachina, M.D. Induction of Trefoil Factor (TFF)1, TFF2 and TFF3 by Hypoxia Is Mediated by Hypoxia Inducible Factor-1: Implications for Gastric Mucosal Healing. *British Journal of Pharmacology* **2009**, *156*, 262–272, doi:10.1111/j.1476-5381.2008.00044.x.
48. Kung-Chun Chiu, D.; Pui-Wah Tse, A.; Law, C.T.; Ming-Jing Xu, I.; Lee, D.; Chen, M.; Kit-Ho Lai, R.; Wai-Hin Yuen, V.; Wing-Sum Cheu, J.; Wai-Hung Ho, D.; et al. Hypoxia Regulates the Mitochondrial Activity of Hepatocellular Carcinoma Cells through HIF/HEY1/PINK1 Pathway. *Cell Death and Disease* **2019**, *10*, 934, doi:10.1038/s41419-019-2155-3.
49. Richardson, S.M.; Knowles, R.; Tyler, J.; Mobasheri, A.; Hoyland, J.A. Expression of Glucose Transporters GLUT-1, GLUT-3, GLUT-9 and HIF-1α in Normal and Degenerate Human Intervertebral Disc. *Histochemistry and Cell Biology* **2008**, *129*, 503–511, doi:10.1007/s00418-007-0372-9.
50. Kim, J.W.; Tchernyshyov, I.; Semenza, G.L.; Dang, C. v. HIF-1-Mediated Expression of Pyruvate Dehydrogenase Kinase: A Metabolic Switch Required for Cellular Adaptation to Hypoxia. *Cell Metabolism* **2006**, *3*, 177–185, doi:10.1016/j.cmet.2006.02.002.
51. Wykoff, C.C.; Beasley, N.J.P.; Watson, P.H.; Turner, K.J.; Pastorek, J.; Sibtain, A.; Wilson, G.D.; Turley, H.; Talks, K.L.; Maxwell, P.H.; et al. Hypoxia-Inducible Expression of Tumor-Associated Carbonic Anhydrases 1. *Cancer Research* **2000**, *60*, 7075–7083.
52. Nishi, H.; Nakada, T.; Kyo, S.; Inoue, M.; Shay, J.W.; Isaka, K. Hypoxia-Inducible Factor 1 Mediates Upregulation of Telomerase (HTERT). *Molecular and Cellular Biology* **2004**, *24*, 6076–6083, doi:10.1128/mcb.24.13.6076-6083.2004.
53. Millhorn, D.E.; Raymond, R.; Conforti, L.; Zhu, W.; Beitner-Johnson, D.; Filisko, T.; Genter, M.B.; Kobayashi, S.; Peng, M. Regulation of Gene Expression for Tyrosine Hydroxylase in Oxygen Sensitive Cells by Hypoxia Regulation of Gene Expression for Tyrosine Hydroxylase in Oxygen. *Kidney International* **1997**, *51*, 527–535.
54. Guo, K.; Searfoss, G.; Krolikowski, D.; Pagnoni, M.; Franks, C.; Clark, K.; Yu, K.T.; Jaye, M.; Ivashchenko, Y. Hypoxia Induces the Expression of the Pro-Apoptotic Gene BNIP3. *Cell Death and Differentiation* **2001**, *8*, 367–376.
55. Feldser, D.; Agani, F.; Iyer, N.; Pak, B.; Ferreira, G.; Semenza, G.L. Reciprocal Positive Regulation of Hypoxia-Inducible Factor 1α and Insulin-like Growth Factor 2. *Cancer Research* **1999**, *59*, 3915–3918.
56. Duś-Szachniewicz, K.; Gdesz-Birula, K.; Zduniak, K.; Wiśniewski, J.R. Proteomic-Based Analysis of Hypoxia-and Physioxia-Responsive Proteins and Pathways in Diffuse Large B-Cell Lymphoma. *Cells* **2021**, *10*, doi:10.3390/cells10082025.
57. Lee, P.J.; Choi, A.M.K. Pathways of Cell Signaling in Hyperoxia. *Free Radical Biology and Medicine* **2003**, *35*, 341–350, doi:10.1016/S0891-5849(03)00279-X.
58. Logothetis, C.J.; Lin, S.H. Osteoblasts in Prostate Cancer Metastasis to Bone. *Nature Reviews Cancer* 2005, *5*, 21–28.

59. DiProspero, T.J.; Dalrymple, E.; Lockett, M.R. Physiologically Relevant Oxygen Tensions Differentially Regulate Hepatotoxic Responses in HepG2 Cells. *Toxicology in Vitro* **2021**, *74*, doi:10.1016/j.tiv.2021.105156.
60. Zhang, H.; Gao, P.; Fukuda, R.; Kumar, G.; Krishnamachary, B.; Zeller, K.I.; Dang, C.V. v.; Semenza, G.L. HIF-1 Inhibits Mitochondrial Biogenesis and Cellular Respiration in VHL-Deficient Renal Cell Carcinoma by Repression of C-MYC Activity. *Cancer Cell* **2007**, *11*, 407–420, doi:10.1016/j.ccr.2007.04.001.
61. Kuo, C.W.; Tsai, M.H.; Lin, T.K.; Tiao, M.M.; Wang, P.W.; Chuang, J.H.; Chen, S. der; Liou, C.W. MtDNA as a Mediator for Expression of Hypoxia-Inducible Factor 1$\alpha$ and ROS in Hypoxic Neuroblastoma Cells. *International Journal of Molecular Sciences* **2017**, *18*, 1220, doi:10.3390/ijms18061220.
62. Tiede, L.M.; Cook, E.A.; Morsey, B.; Fox, H.S. Oxygen Matters: Tissue Culture Oxygen Levels Affect Mitochondrial Function and Structure as Well as Responses to HIV Viroproteins. *Cell Death and Disease* **2011**, *2*, e246, doi:10.1038/cddis.2011.128.