HIF-2 Regulation of Amino Acid Transporter Expression in Hepatocellular Carcinoma Cells

Jason A. Misurelli
jason.misurelli@gmail.com

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ABSTRACT

HIF-2 REGULATION OF AMINO ACID TRANSPORTER EXPRESSION IN HEPATOCELLULAR CARCINOMA CELLS

Jason A. Misurelli, M.S.
Department of Biological Sciences
Northern Illinois University, 2019
Barrie P. Bode, Director

Hypoxia represents a major driver of the cancer phenotype in solid tumors, especially those of hepatic origin. Low oxygen availability triggers a complex cellular response which alters metabolism and promotes cellular invasion. Growing evidence suggests the amino acid glutamine, an important molecular currency for both energy-producing reactions and biosynthetic pathways, plays an enhanced role in cancer cells under hypoxia. Hypoxia-inducible factors (HIFs) are transcription factors crucial for regulating the cellular response to low oxygen. Family member HIF-1 has been well studied; however, recent evidence suggests HIF-2 may have a regulatory role in amino acid transport and metabolism under hypoxia. Therefore, this study generated a series of human liver cancer cell lines either lacking or overexpressing the HIF-2 alpha subunit in order to examine its potential role in cellular glutamine transport. The expression of amino acid transporters ASCT2 and LAT1 was examined under both normal and low oxygen conditions. Loss of HIF-2α resulted in a substantial decrease in ASCT2 under long-term hypoxia but did not appear to impact LAT1. No change in either transporter
was observed with the overexpression of HIF-2α. Results suggest HIF-2 may be necessary to maintain hypoxic expression of certain c-Myc-regulated genes, possibly by countering the inhibitory effect of HIF-1. The broader role of HIF-2 on amino acid transport, including transporters not driven by Myc, will require further investigation.
HIF-2 REGULATION OF AMINO ACID TRANSPORTER EXPRESSION IN
HEPATOCELLULAR CARCINOMA CELLS

BY
JASON A. MISURELLI
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A THESIS SUBMITTED TO THE GRADUATE SCHOOL
IN PARTIAL FUFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE
MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

Thesis Director:
Barrie P. Bode
ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Barrie Bode for welcoming me into his lab. His knowledge and guidance have been invaluable during my graduate career and I have been so fortunate to have him as a mentor. I would also like to thank the other members of my committee, Dr. Olivier Devergne and Dr. Linda Yasui for their time, insights and input on my project.

I am also very grateful to Dr. Paige Bothwell for all her assistance on everything from western blots to microscopy and Dr. Clare Kron for her help with qPCR techniques. A special thanks to the past and present members of the Bode Lab I’ve had the chance to work with including Aparna Agarwal, Doaa Alshammari and Stephanie Cooper Woodin.

Finally, I would like to thank my mother, Edith Misurelli, and my father, Joseph Misurelli, for all their love and support that helped me reach this point.
DEDICATION

I dedicate this thesis to my father, Joseph Misurelli.
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INTRODUCTION

_Hypoxia_. Tissues throughout the human body are subjected to a range of oxygen tensions; however, when their environment becomes hypoxic, an adaptive response is triggered. This response is recognized in virtually all types of cells and involves hundreds of genes which initiate alterations in energy metabolism, cell migration, angiogenesis, pH regulation, proliferation and survival (Tennant et al., 2009). Hypoxia-inducible factors (HIFs) are the primary modulators of the hypoxic response. These helix-loop-helix transcription factors bind target genes possessing hypoxia response elements (HREs) that contain G/ACGTG sequences (Schofield & Ratcliffe, 2004). Each HIF is a heterodimer composed of both an α and β subunit. HIFβ, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed, while HIFα is regulated at the protein level by efficient degradation (Figure 1) under normoxic conditions (Huang, Li, & Zhang, 2014). This mechanism is initiated by α-ketoglutarate-dependent prolyl hydroxylases (PHDs) which hydroxylate Pro564 or Pro531 on HIFα subunits, a process also requiring Fe(II) and oxygen as cofactors (Schofield & Ratcliffe, 2004). Once hydroxylated, HIFα subunits are targeted for ubiquitination by the von Hippel–Lindau tumor suppressor (pVHL) containing E3 ubiquitin ligase complex, ultimately leading to proteasomal degradation. Under hypoxic conditions, without oxygen as a cofactor, PHDs are inactivated. The stabilization of HIFα subunits allows cellular accumulation and dimerization with HIFβ and, along with coactivators CBP/p300, translocate to the
nucleus to initiate transcription of HIF target genes (Ratcliff, 2007). This protein-level regulatory mechanism allows for a rapid and graded response to aberrations in oxygen availability within a cell.

**Figure 1: Regulation of hypoxia-inducible factors.** The alpha subunit of HIF proteins is rapidly degraded under adequate oxygen (normoxia) but stabilizes and forms its transcriptional complex under low oxygen (hypoxia).

**HIF-1 and HIF-2.** To date, three different HIFα isoforms have been identified: HIF-1α, HIF-2α and HIF-3α. HIF-3α is the most recently discovered and least characterized isoform, but lacking a transactivation domain, it is believed to be a
repressor of HRE-containing genes (Courtnay et al., 2015). HIF-1α and HIF-2α are closely related, with an 85% sequence similarity and share overlapping functions, but also display nonredundant roles (Singh et al., 2017). HIF-1α is ubiquitously expressed in all tissues and was the first HIF to be identified (Huang, Li, & Zhang, 2014). In addition to angiogenesis, HIF-1 is recognized as the major driver in the shift from oxidative metabolism to glycolysis under hypoxia by upregulating lactate dehydrogenase A (LDHA) and pyruvate dehydrogenasekinase-1 (PDK1) to shunt pyruvate away from entering the TCA cycle and toward lactate formation (Tennant et al., 2009). Increased expression of glucose transporters GLUT1 and GLUT3 are also driven by HIF-1, enhancing glucose uptake and further supporting glycolytic metabolism (Huang, Li, Li, et al., 2014). Intracellular pH is maintained in response to the excess H+ generated from increased glycolysis by HIF-1-induced expression of monocarboxylate transporter 4 (MCT4) and sodium-hydrogen antiporter 1 (NHE1; Tennant et al., 2009). A glycolytic phenotype is further supported by HIF-1 through the downregulation of complex 1 and complex 2 in the electron transport chain (Nakazawa, Keith, & Simon, 2016). HIF-1 can additionally attenuate oxidative metabolism by inducing mitophagy through the expression of the proapoptotic Bcl-2 protein BNIP3 (Masson & Ratcliffe, 2014).

In contrast, the more recently discovered HIF-2α exhibits more tissue-specific expression in blood vessels and organs including the liver, heart, kidney, brain and lungs (Holmquist-Mengelbier et al., 2006). HIF-2 acts as a transcription factor for multiple prosurvival factors such as VEGF, TGF-α and cyclin D1 (Ratcliffe, 2007). HIF-2 also
appears to support lipid accumulation and inhibits fatty acid oxidation by suppressing carnitine palmitoyltransferase I (Cpt-1) and acyl-CoA synthase long-chain family member 1 (Acs1l1; Huang, Li, Li, et al., 2014). One notable difference between HIF-1 and HIF-2 transcription factors is that HIF-1 is a repressor of Myc via MXI1 induction and an inhibitor of mTORC1 through the activation of TSC1/TSC2 whereas HIF-2 has been shown to be an activator of both Myc and mTORC1 activity (Elorza et al., 2012).

In addition to unique targets, the HIFs also display differences in regulation. Transactivation of stabilized HIFα can be prevented by factor inhibiting HIF (FIH-1), an oxygen-dependent asparaginyl hydroxylase targeting the asparaginyl residue Asn803 of HIF-1α or Asn851 of HIF-2α, preventing interaction with its co-activator p300 (Schofield & Ratcliffe, 2004). Due to the C-terminal sequence differences between them, FIH-1 hydroxylates HIF-1α with a greater efficiency than HIF-2α, leading to higher levels of transcriptionally active HIF-2 (Koivunen et al., 2003). While expression patterns of HIF-1α and HIF-2α can be variable depending on the tissue, HIF-2α has been observed to begin stabilizing under 5% oxygen in liver and brain tissue while HIF-1α does not appreciably stabilize until more severe conditions of 1% oxygen (Bracken et al., 2006). HIF-2α also appears to accumulate more gradually and continues to be present after 72 hours of hypoxia whereas HIF-1α accumulates rapidly within 2-8 hours before degradation (Holmquist-Mengelbier et al., 2006). These differences highlight the nonredundant roles of the HIF factors and suggest that HIF-1 may be expressed as an
immediate response to acute hypoxia while HIF-2 may mediate the cellular response to more prolonged hypoxia.

As the master regulators of the hypoxic response, HIFs have become a target of interest in cancer research given that hypoxia within the tumor microenvironment is a frequent feature of cancer (Li et al., 2017). This is especially true in solid tumors where rapidly proliferating cells form masses which lose access to a sufficient blood supply, leading to hypoxia and ultimately initiating a hypoxic response in the affected tissues. The resulting changes in metabolism, in addition to the activation of pro-survival and tissue remodeling pathways, contribute to a malignant phenotype (Singh et al., 2017).

Even under normoxic conditions, tumors have been observed to express HIF proteins, contributing to the so-called “Warburg effect” where cancer cells display aerobic glycolysis as a primary source of energy (Perez-Escuredo et al., 2015). HIF stabilization in the presence of adequate oxygen can occur through multiple mechanisms including genetic mutation leading to loss of VHL or accumulation of metabolites such as lactate that inactivate PHDs through competition with co-factor α-ketoglutarate (Polet & Feron, 2013).

The upregulation of HIFs in tumors has been correlated with poor prognosis and worse patient outcomes in cancers such as hepatocellular carcinoma (HCC; Nath & Szabo, 2012). VHL-deficient renal cell carcinoma (RCC) xenografts that express HIF-1α alone have not been observed to form tumors while those expressing HIF-2α alone formed tumors that were larger and more rapidly proliferating than xenografts expressing
both isoforms (Raval et al., 2005). Inhibition of HIF-2α is also sufficient to suppresses VHL-deficient RCC tumor formation in vivo (Kondo et al., 2003). Such observations, in light of HIF-2 as an activator of both Myc and mTOR pathways, have led to the hypothesis that HIF-2 may play pro-oncogenic roles while HIF-1 may exhibit more anti-oncogenic effects. Considering the genes and processes they mediate, HIFs are attractive therapeutic targets. However, effective HIF-directed therapies will require an understanding of the differential roles each HIF transcription factor plays in tumorigenesis, especially when targeting one specific HIF isoform.

**Glutamine Metabolism.** The purpose of this project is to examine the role of HIF-2 on glutamine metabolism using liver cancer as a model. As a driver of glycolysis, research investigating the link between HIFs and cancer has largely focused on HIF-1 and the role of glucose metabolism (Kondo et al., 2003). However, both glutamine and glucose serve as the two major sources of energy and carbon in cancer cells and more recent studies highlight the relationship between hypoxia and glutamine metabolism (Courtnay et al., 2015).

Glutamine, the most abundant amino acid in circulation, serves a variety of crucial roles (Figure 2) in energy production, biosynthesis and cell survival (Lukey, Wilson, & Cerione, 2013). Consumption of glutamine is significantly increased in malignancies as cancers rely on these pathways to support survival and proliferation (Marchiq & Pouysségur, 2015). Glutamine can be utilized for energy by undergoing
Figure 2: The major cellular roles of glutamine. The amino acid glutamine is involved in multiple anapleurotic and biosynthetic pathways supporting cell function.
glutaminolysis, supplying the TCA cycle with α-ketoglutarate to yield ATP. Glutamine can also feed glycolysis with glucose by serving as a starting material for gluconeogenesis (Michalak et al., 2015). In cancer cells, glycolysis often becomes uncoupled from the TCA cycle as glucose-derived pyruvate is shunted to lactate formation rather than acetyl-CoA (Corbet & Feron, 2015). Highly glycolytic cells therefore require glutamine to supplement the TCA cycle intermediates and feed biosynthetic pathways necessary in supporting rapidly proliferating cells. Using stable isotope resolved metabolomics, Le et al. (2012) observed that malignant B cells displayed a Myc-driven increase in glutamine metabolism and utilization under hypoxia. This glucose-independent TCA cycle pathway used glutamine as a carbon source for both biogenesis and energy. This pathway efficiently generated 17.5 mol ATP per mol glutamine versus the 5 mol ATP generated by the typical glutaminolysis pathway, where glutamine is converted to lactate rather than oxidation through the TCA cycle. The crucial role of glutamine in supporting this malignant phenotype was demonstrated when inhibition of glutaminase effectively killed hypoxic cancer cells in vitro and delayed the growth of xenograft tumors in vivo.

Rapidly proliferating cancer cells need to maintain multiple biosynthetic pathways to supply lipids, nucleic acids and proteins necessary for growth and reproduction. Glutamine can be converted to glutamate, then to α-ketoglutarate, where it can pursue two different routes in lipid synthesis: the (forward) oxidative decarboxylation pathway to generate acetyl-CoA or, more frequently in hypoxic cancer cells, the (reverse)
reductive carboxylation pathway to generate acetyl-CoA from citrate (Filipp et al., 2012). While HIF-2α promotes fatty acid accumulation by regulating Cpt-1 and Acs11, HIF-1 supports de novo lipogenesis under hypoxia through reductive metabolism of glutamine by regulation of IDH2 (Huang, Li, & Zhang, 2014). Under hypoxia, the use of glutamine-derived acetyl-CoA for lipogenesis increases from 10-25%, normally seen under normoxia, up to 80% (Daye & Wellen, 2012). Glutamine also serves as an important role in nucleotide biosynthesis as a nitrogen source for purine and pyrimidine as well as a carbon source through glutamine-derived aspartate via the TCA cycle (Altman, Stine, & Dang, 2016).

As a nitrogen donor, glutamine can synthesize multiple nonessential amino acids including alanine, aspartate, serine, and asparagine via transamination reactions or be directly converted to proline. Cancer cells rely on glutamine as a direct source of at least 50% of nonessential amino acids for protein synthesis (Tarrado-Castellarnau, Atauri, & Cascante, 2016). Glutamine further maintains the amino acid pool by its exchange through antiporters for essential amino acids.

**ASCT2 and LAT1.** In malignancies, amino acid transporters ASCT2 (SLC1A5) and LAT1 (SLC7A5) are often highly expressed together, especially those with an aggressive phenotype (Fuchs & Bode, 2005). ASCT2 is a sodium-dependent, neutral amino acid transporter which serves as a major source of nonessential amino acid uptake, including glutamine, into the cell. LAT1 is a sodium-independent exchanger of
nonessential amino acids for essential amino acids. The coupling of ASCT2 and LAT1 allows for the import of glutamine by ASCT2 which in turn is exchanged for essential amino acids by LAT1 (Bhutia et al., 2015). This represents an important mechanism for glutamine-addicted cells to take up mTOR-activating amino acids like leucine to promote growth and inhibit apoptosis (Chen & Cui, 2015). The importance of glutamine can be seen in cancer cells expressing high levels of c-Myc, a promoter of both ASCT2 and LAT1, where withdrawal of glutamine induces cell death (Daye & Wellen, 2012).

Multiple studies have found evidence that HIF-2α is capable of influencing both ASCT2 and LAT1 expression. In SiHa and HeLa cells, Perez-Escuredo et al. (2015) demonstrated that lactate-driven inhibition of PHDs allows for the accumulation of HIF-2α, which in turn enhances c-Myc activity by stabilizing c-Myc/Max interaction. This upregulation of c-Myc increases expression of ASCT2 and glutaminase (GLS1), the enzyme which converts glutamine to glutamate. Elorza et al. (2012) showed that in VHL-deficient tumor cells, HIF-2α is able to bind the proximal promotor of LAT1. This upregulation of LAT1 was found to increase mTORC1 activity, supporting a proliferative phenotype in tumors and opposing the HIF-1-driven decrease in mTORC1 occurring under low oxygen. Corbet et al. (2014) observed similar results in SiHa cells exposed to chronic acidosis. Under an acidic pH of 6.5, SIRT1 deacetylase reduced HIF-1α activity and increased HIF-2α activity, upregulating both LAT1 and GLS1 along with an increase in reductive glutamine metabolism.
**xCT and ROS.** Glutamine also plays a crucial role in redox maintenance by synthesis of glutathione, a Glu-Cys-Gly tripeptide. Glutathione has multiple cellular functions including DNA repair, cell cycle regulation and neutralization of reactive oxygen species (ROS) such as peroxide and superoxide free radicals (Michalak et al., 2015). Cancer cells display higher levels of active glutathione, contributing to higher rates of proliferation and chemotherapy resistance (Daye & Wellen, 2012). Glutamine is not only necessary as a component of glutathione but also indirectly, as a means of cysteine. Glutamate, derived from glutamine, is exchanged through the amino acid antiporter xCT (encoded by the *SLC7A11* gene and its non-catalytic chaperone 4F2 heavy chain, encoded by *SLC3A2*) for cystine, which is then reduced to cysteine (Bhutia et al., 2015). Additionally, glutamine is a source of aspartate and malate which, upon conversion to pyruvate, generates NADPH needed to recycle glutathione back to its active form (Kishton & Rathmell, 2015).

Attenuating the ROS generated under limited oxygen is one important function of the hypoxic response. HIF-2α is known to upregulate antioxidant enzymes such as superoxide dismutase (SOD2; Befani et al. 2013). HIF-1, in addition to more suppressive roles of mitochondrial function, also promotes the expression of an alternate cytochrome c oxidase subunit IV (COX-4) isoform which operates more efficiently under low oxygen (Nakazawa, Keith, & Simon, 2016). Likewise, both HIFs appear to support glutamine-based pathways of regulating cellular redox. Li et al. (2017) observed that knockdown of HIF-2α attenuated glutamine consumption in pancreatic ductal adenocarcinoma (PDAC)
and led to increased levels of ROS. HIF-1 has been shown to induce transcription of transporter xCT and glutamate-cysteine ligase regulatory subunit (GCLM), increasing glutathione synthesis in breast cancer stem cells (Parks et al., 2016).

**Targeting HIF-2α.** Taken together, there is increasing evidence that suggests a relationship between HIF transcription factors and multiple aspects of glutamine metabolism in cancer. This relationship with HIF-2α appears to potentially support a pro-oncogenic phenotype and metabolic reprogramming through pathways involving Myc and mTOR. The role of HIF-1α, a negative regulator of both pathways, is less apparent, however. This project explores the specific role of HIF-2 by altering its expression in a hepatocellular carcinoma cell line (Huh-7). Stable cell lines were generated where the HIF-2α subunit is either overexpressed or knocked out using CRISPR-Cas9. The CRISPR-Cas9 system (Figure 3) involves both the Cas9 enzyme and a specific gRNA sequence targeting the gene of interest. Once recognized, the enzyme introduces a double-stranded break, which will cause either homologous recombination or (more likely) non-homologous end joining (NHEJ) that results in a possibly nonfunctional gene product. This method is advantageous in providing a complete gene knockout instead of a knockdown, creating a cell model which expresses only functional HIF-1 and HIF-3 hypoxia-inducible transcription factors.
Figure 3: CRISPR-Cas9-mediated gene knockout. The target gene is recognized by a specific gRNA sequence, initiating a cut to both DNA strands by the Cas9 endonuclease. The resulting repair mechanism creates a possibly nonfunctional gene product.

Both the redundancy and specialized roles of HIF-1α and HIF-2α result in a complex system responsible for mediating the hypoxic response while also capable of driving a malignant phenotype. Further description of the differential roles of each HIF transcription factor will inform a more complete model of tumorigenesis under hypoxia and other HIF-stabilizing environments. This understanding, in turn, can inform more effective treatment strategies and potentially improve patient outcomes in the long term.
MATERIALS AND METHODS

Cell Culture. The human hepatocellular carcinoma cell line Huh-7 used in this project was obtained from Dr. J. Liang, Massachusetts General Hospital (MGH). Parent and transfected cell lines were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in a HERAcell 150i incubator (Thermo Fisher Scientific, Rochester, NY). Hypoxia was simulated at 1% O₂ using a NAPC Series 8000 WJ cell culture incubator (Thermo Fisher Scientific, Rochester, NY) purged with nitrogen gas. Cells were cultured in BioLite 25cm vented flasks (Thermo Fisher Scientific, Rochester, NY) and experiments were performed in Costar six-well, 12-well, and Falcon 100mm cell culture plates (Corning Incorporated, Corning, NY). Cells were grown in high glucose (4.5g/L) Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 2mM L-glutamine, 1% antibiotic-antimycotic solution (10,000 U/ml penicillin G, 10,000 µg/ml streptomycin, and 25 µg/ml amphotericin as a 100x concentration; all from Gibco, Grand Island, NY) and 10% triple-filtered fetal bovine serum (FBS; GE Lifesciences, Logan, Utah). Transfected cell lines were maintained in medium with added puromycin (Gibco, Grand Island, NY) at a concentration of 3µg/mL. Cell counts were performed using a standard bright-line hemacytometer (Hausser Scientific, Horsham, PA) and a Nikon Diaphot phase contrast microscope (Frank E. Fryer Co., Carpentersville, IL).
**Plasmid Constructs.** HIF-2α knockout lines were generated using CRISPR all-in-one vectors (Figure 4) purchased from transOMIC Technologies, Inc. (Huntsville, AL) containing either a non-targeting gRNA sequence or one of three EPAS1 (HIF-2α) targeted sequences (Table 1). The HIF-2α overexpression vector pCDH-CB1-HIF2α-GFP-T2A-Puro (Figure 5) was a gift from Eric Jonasch and Xiande Liu (Addgene plasmid # 71708; http://n2t.net/addgene:71708; RRID:Addgene_71708). The corresponding empty vector pCDH-CB-IRES-copGFP-T2A-Puro (Figure 6) was a gift from Kazuhiro Oka (Addgene plasmid # 72299; http://n2t.net/addgene:72299; RRID:Addgene_72299). Stock plasmid-expressing bacterial cultures were streak plated on agar supplemented with 100 μg/μL ampicillin (Fisher Scientific, Fair Lawn, NJ). Isolated colonies were grown out in LB broth with the same antibiotic concentration and used to inoculate 50mL LB cultures at 37°C and 110 RPM for 21 hours. Bacterial cultures were harvested and plasmid DNA was purified according to the protocol supplied with PureYield Plasmid Midiprep System (Promega, Madison, WI). Plasmid DNA was resuspended in 500 μL of nuclease-free water and quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). Cryovials of bacterial stocks were stored in 50% LB broth / 50% glycerol at -80°C. Stocks of purified plasmid DNA were stored at -20°C.
Figure 4: Plasmid map of pCLIP vector for gRNA and Cas9 expression (transOMIC Technologies, Inc.).

Table 1: gRNA Sequences for HIF-2α Knockouts and Non-Silencing control

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<th>Name</th>
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<td>EPAS1</td>
<td>TEVH-1077411</td>
<td>GTCTCAGGTCTTGCACTGCA</td>
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<tr>
<td>B</td>
<td>EPAS1</td>
<td>TEVH-1144553</td>
<td>ACCAAGGGTCAGGTAGTAAG</td>
</tr>
<tr>
<td>C</td>
<td>EPAS1</td>
<td>TEVH-1211695</td>
<td>ACTGGCACCCTATATCCCCA</td>
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<td>NS</td>
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Figure 5: Plasmid map for pCDH expression vector containing GFP-tagged HIF-2α construct (Addgene).
Figure 6: Plasmid map of pCDH empty vector expressing GFP (Addgene).
**Transfection.** Huh-7 cells were seeded at a density of 1x10^5 cells in 1mL of DMEM in triplicate wells of a 24-well plate. After 24 hours of incubation, 100 μL of medium containing 1μg of plasmid DNA and 2 μL TurboFect *in vitro* transfection reagent (Thermo Scientific, Waltham, MA) were added to each well. After 24 hours, selective medium was introduced containing 3 μg/mL puromycin, an effective dose determined by previous kill curve experiments. Selection for transfected cells was carried out over the course of 14 days with replacement of medium/antibiotic every three days. When near confluent, low-passage cells were frozen back in liquid nitrogen storage at a concentration of 10x10^6 cells per 1 mL medium supplemented with 5% dimethyl sulfoxide (DMSO) in cryovials.

**Microscopy.** Microscopic imaging of fluorescent protein was performed with a Zeiss LSM 5 Pascal Confocal Laser Scanning Microscope with 40x oil immersion objective lens and using a HFT88 dichroic mirror and LP505 emission filter. Cells were seeded at a concentration of 0.1x10^6 on #1 (0.13 -0.16 mm) sterile glass coverslips in a six-well plate and incubated under their respective oxygen conditions for 48 hours. Cells were then washed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde (PFA) for 30 minutes before transfer to a standard microscope slide with a mounting solution to preserve fluorescence containing 100 mg/mL 1,4-diazabicyclo[2.2.2]octane (DABCO) in 50% PBS / 50% glycerol.
**RT-qPCR.** Cells were harvested with TRIzol Reagent (Life Technologies, Grand Island, NY), and RNA isolation was performed according to the manufacturer’s protocol. RNA pellets were resuspended in nuclease-free water and quantified using a Nanodrop 2000 Spectrophotometer. cDNA synthesis was performed using an ImProm-II Reverse Transcription System kit (Promega, Madison, WI) according to manufacturer’s protocol with an RNA template concentration of 50ng/μL per reaction. qPCR was performed using TaqMan Gene Expression Assays (Life Technologies Corporation, Pleasanton, CA) for ASCT2, LAT1 and reference gene YWHAB, selected based on expression stability in the Huh-7 cell line under hypoxia (Liu et al., 2017) with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) according to manufacturer’s protocol in triplicate with fast 96-well plates (Thermo Scientific, Waltham, MA). Reverse transcription and PCR reactions were run using an Eppendorf realplex² Mastercycler (Eppendorf, Hauppauge, NY). Results were analyzed using the delta-delta Ct method, and statistical significance between each knockout or overexpression sample to its respective control was determined using one-way ANOVA or t test.

**Western Blot Analysis.** Cells were harvested in lysis buffer (8% w/v SDS, 250 mM tris HCl, pH 6.8) and protein concentration was determined by UV-Vis with a Nanodrop 2000 Spectrophotometer. Loading samples were prepared at a concentration of
1.3 μg/μL of total protein with 10% glycerol, 0.025% w/v bromophenol blue and 50 mM dithiothreitol (DTT). Samples were electrophoresed through 4-20% Mini-PROTEAN TGX polyacrylamide gels (Bio-Rad, Hercules, CA) at 150V for 45 minutes at room temperature. Protein samples (20μg per lane) were loaded in each lane. PageRuler Plus Prestained Protein Ladder (Thermo Scientific, Waltham, MA) and Biotinylated Protein Ladder (Cell Signaling, Danvers, MA) were loaded as molecular weight markers.

Membrane transfer was performed at 75V for 90 minutes on Immobilon polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were blocked with 5% bovine album serum (BSA; Gold Biotechnology, St. Louis, MO) in 95% TBST (0.1% Tween-20 in tris-buffered saline at pH 7.6). PVDF membranes were incubated overnight at 4°C with primary antibody stocks prepared at 1:1000 dilution in blocking buffer. Following incubation, blots received three washes of TBST and 1 hour of incubation at room temperature with HRP-conjugated second secondary antibody and anti-biotin antibody (both from Cell Signaling, Danvers, MA) prepared at 1:2000 dilution in blocking buffer. Blot received three additional washes of TBST before development with LumiGLO and peroxide reagent (Cell Signaling, Danvers, MA). Images were obtained using a G:BOX Chemiluminescence Imager (Syngene, Frederick, MD). Following imaging, blots were washed and re-probed with HRP-conjugated β-actin antibody at 1:1000 dilution as a loading control. Primary antibodies for HIF-2α, ASCT2, LAT1, c-Myc, and β-Actin were purchased from Cell Signaling (Danvers, MA) and xCT antibody from Pierce (Rockford, IL).
RESULTS

*Evaluation of Stable HIF-2α Knockout Lines.* Human hepatocellular carcinoma (HCC) Huh-7 cells were transfected as described in the materials and methods section with a Cas9 expression vector containing one of three gRNA sequences targeting *EPAS1* (HIF-2α). To distinguish them, these knockout lines were named A, B, and C (see Table 1 for corresponding sequences) and will be referenced this way in the data presented here. As a control, one cell line was generated by transfection with a Cas9 vector containing a non-targeting gRNA sequence and is referred to here as NS for “non-silencing.” After initial selection with puromycin, surviving cell populations were plated at a density of 2.55x10^5 cells per well in a six-well plate and incubated for 24 hours under either normoxia (atmosphere supplemented with 5% CO₂), hypoxia (1% O₂) or 18 hours normoxia followed by 6 hours hypoxia. Cell lysates were analyzed by western blot for HIF-2α protein expression (Figure 7).
Figure 7: Confirmation of HIF-2α gene knockout. Western blot showing HIF-2α expression in Huh-7 cell lines non-silencing control (NS) and HIF-2α knockout lines (A, B & C) at different intervals of hypoxia (1% O₂). (n=1)

Results show a faint band present in NS control under 0 hours of hypoxia and a clear presence of HIF-2α after only 6 hours of hypoxia which is sustained after 24 hours of hypoxia. By contrast, none of the three HIF-2α knockout lines show HIF-2α protein expression even under hypoxia. This suggests all three gRNA sequences were effective in silencing the EPAS1 gene.

Evaluation of Stable HIF-2α Overexpressing Lines. Huh-7 cells were transfected with plasmids either expressing GFP-tagged HIF-2α, referred to here as “overexpressing” (OE), or an empty vector (EV) containing genes for puromycin resistance and GFP expression but lacking the gene of interest. After selection with puromycin, surviving cell lines along with untransfected Huh-7 cells (referred to here as P for parent) were incubated under different intervals of hypoxia, repeating the same experimental setup as previously
described for the knockout cell lines. Cell lysates were harvested and examined by western blot (Figure 8).

![Western blot showing HIF-2α overexpression](image)

**Figure 8: Conformation of HIF-2α overexpression.** Western blot showing HIF-2α expression in Huh-7 parent line (P), empty vector pCDH line (EV) and HIF-2α overexpressing line (OE) at different intervals of hypoxia (1% O₂). (n=1)

Very faint bands for HIF-2α are present for all three cell lines when cultured at normoxic conditions. Protein accumulation becomes clear after 6 hours and persists after 24 hours of 1% oxygen. The overexpressing line shows two sets of bands, the lower being endogenous HIF-2α at around 120 kDa while the higher molecular weight band is the transfected HIF-2α gene product containing the addition of GFP (~27 kDa). This data confirms the successful generation of a Huh-7 cell expressing the HIF-2α-GFP fusion protein.

The GFP tag on HIF-2α in the overexpressing line allows for the opportunity to visualize the cellular distribution of the protein under different conditions.
To do this, cells were plated at a density of $1 \times 10^5$ on coverslips in six-well plates and incubated at 37°C over the course of 48 hours. Cells were fixed to prevent HIF protein degradation when exposed to oxygenated atmosphere and then mounted for fluorescent microscopy. Cells were exposed to either 48 hours of normoxia, 6 hours of hypoxia, 24 hours of hypoxia, or 48 hours of hypoxia (Figure 9). Cells incubated under only normoxia show expression of GFP-tagged HIF-2α, which appears entirely outside the nucleus. With a duration of 6 hours at hypoxic conditions, nuclear HIF-2α is visible, and under longer periods of 24 and 48 hours, accumulation increases.

Groups treated with cobalt chloride, a chemical mimic of hypoxic, for 24 hours (Figure 10) display a stronger response compared to even the longest treatment with 1% O$_2$. A relatively low dose of 50μM resulted in clear nuclear accumulation of HIF-2α. Treatment with a higher dose of 150μM produced substantially more accumulation by comparison.
Figure 9: Cellular HIF-2α expression as a result of low oxygen. GFP-tagged HIF-2α expressed at normoxia (A), 6 hrs 1% O₂ (B), 24 hrs 1% O₂ (C) and 48 hrs 1% O₂ (D) at 400x magnification. Fluorescence (left), brightfield (right) and overlay (middle). (n=1)
Figure 10: Subcellular distribution of HIF-2α in response to CoCl₂ concentrations. GFP-tagged HIF-2α expression in response to exposure to 0μM (A), 50μM (B), and 150μM (C) for 24 hrs at 400x magnification. Fluorescence (left), brightfield (right) and overlay (middle). (n=1)
**Effect of HIF-2 on ASCT2.** With the establishment of both HIF-2α knockout and overexpressing Huh-7 cell lines, different intervals of hypoxia were screened using these manipulated cell lines to identify any interaction between ASCT2 and HIF-2. In order to directly compare four different time points on one blot, only the non-silencing and two knockout lines (A & B) were chosen for the initial screening. Cells were plated at a density of 1 x 10^5 per well of a six-well plate and incubated a total of 48 hours under either normoxic conditions, 6 hours hypoxia, 24 hours hypoxia or 48 hours hypoxia (Figure 11). Results showed ASCT2 expression in HIF-2α knockout lines were relatively consistent with the non-silencing control under normoxia and 6 hours of hypoxia. However, between 24 hours and 48 hours of hypoxia, there appeared to be a decrease in ASCT2 in the knockout lines, especially line B, compared to the control. This suggested that long-term hypoxia at the 48-hour window was a time point where HIF-2 may influence ASCT2 expression levels.
Figure 1: ASCT2 expression under increasing intervals of hypoxia. Western blot showing Huh-7 non-silencing (NS) and HIF-2α knockout lines (A & B) at different periods of hypoxia (1% O₂). (n=1)

The experiment was repeated with all three knockout lines, the non-silencing control and the parent Huh-7 line comparing only 48 hours normoxia vs. 48 hours hypoxia (Figure 12). The parent line showed the highest ASCT2 expression under normoxia while the non-silencing and HIF-2α knockout lines showed similar levels of expression. Under 48 hours of hypoxia, both parent and NS lines maintained ASCT2 expression near to that of normoxia. However, all three HIF-2α knockout lines showed a clear decrease, especially lines B and C. The difference between normoxic and hypoxic ASCT2 expression was demonstrated to be statistically significant in all three knockouts compared to their non-silencing control.
Figure 12: HIF-2 maintains ASCT2 under long-term hypoxia. Western blot showing Huh-7 parent (P), non-silencing (NS) and HIF-2α knockout lines (A, B, & C) under either 48 hrs. normoxia or hypoxia (1% O₂) (A). Densitometry showing relative ASCT2 protein quantification (as a ratio of β-actin and normalized to NS normoxia) between cell lines. Differences between hypoxic ASCT2 expression NS vs. A *** (p=0.0005), NS vs. B *** (p = 0.0002), NS vs. C ** (p = 0.0030) determined by one-way ANOVA (B). Continued on following page.
Figure 12 continued. Densitometry showing percent change in ASCT2 expression between normoxic and hypoxic conditions in each cell line. NS vs. P ns (p = 0.8205), NS vs. A * (p = 0.0474), NS vs. B *** (p = 0.0007), NS vs. C ** (p = 0.0069) (C). (n=3), one-way ANOVA with Holm-Sidak’s test for multiple comparisons, +/- = SEM. Cutoff for p values (ns > 0.5), (* ≤ 0.05), (** ≤ 0.01), (***) ≥ 0.001, (**** ≥ 0.0001).
To evaluate whether differences in ASCT2 between cell lines were occurring at the transcriptional level, RT-qPCR was performed. Cell lines were plated at a density of 6 x 10^5 cells in 100 mm cell culture plates and incubated under either normoxia or hypoxia for 48 hours (Figure 13). Results showed an induction in ASCT2 mRNA expression for both untransfected Huh-7 parent and non-silencing controls under hypoxia. HIF-2α knockout lines showed no induction of ASCT2 expression and mRNA is clearly decreased compared to normoxic expression in A and B lines. Once again, the difference between normoxic and hypoxic ASCT2 was statistically significant in all three knockouts compared to their non-silencing control at the mRNA level.

Experiments were repeated for the HIF-2α overexpressing line. Huh-7 parent lines, empty vector pCDH and HIF-2α overexpressing cells were incubated for 48 hours under either normoxia or hypoxia. Cells were harvested and ASCT2 protein expression was analyzed by western blot (Figure 14). Results show a small increase under normoxia in ASCT2 for the HIF-2α overexpressing lines. However, no difference was observed across all three cell lines under hypoxia.
Figure 13: HIF-2 induces ASCT2 mRNA expression under hypoxia. qPCR showing ASCT2 expression in Huh-7 parent line, non-silencing (NS) and HIF-2α knockout lines (A, B, & C) under normoxia vs. 48 hours hypoxia (1% O2). Differences between hypoxic ASCT2 mRNA expression NS vs. A **** (p=0.0001), NS vs. B **** (p = 0.0001), NS vs. C ****(p = 0.0001) determined by one-way ANOVA (A). Continued on following page.
Figure 13 continued. Percent change in ASCT2 mRNA between normoxic and hypoxic conditions in each cell line. NS vs P ns (p = 0.4812), NS vs. A * (p = 0.0131), NS vs. B ** (p = 0.0034), NS vs. C * (p = 0.0391) (B). (n=3), one-way ANOVA with Holm-Sidak’s test for multiple comparisons, +/- = SEM. Cutoff for p values (ns > 0.5), (* ≤ 0.05), (**) ≤ 0.01), (***) ≥ 0.001), (**** ≥ 0.0001).
Figure 14: HIF-2α overexpression fails to upregulate ASCT2 under hypoxia. Western blot showing Huh-7 parent (P), empty vector pCDH (EV) and HIF-2α overexpressing (OE) cell lines under either 48 hrs normoxia or hypoxia (1% O2). (n=1)

To examine mRNA expression, qPCR was performed following the same setup. Huh-7 parent, empty vector and HIF-2α overexpressing lines were incubated at either 48 hours normoxia or hypoxia (Figure 15). Results show some variation in normoxic and hypoxic expression of ASCT2 mRNA across cell lines but display a consistent pattern of a small induction under hypoxia. Differences between HIF-2α overexpressing cells and their empty vector control were not statistically significant.
**Figure 15: HIF-2α overexpression fails to induce ASCT2 mRNA expression.** qPCR showing Huh-7 parent, empty vector pCDH and HIF-2α overexpressing under 48 hrs of normoxia or hypoxia (1% O₂). Statistical significance determined by t test between hypoxic ASCT2 expression in pCDH and overexpressing line (p = 0.12649). (n=1) +/- = SEM.

**Effect of HIF-2 on LAT1.** Next, the other major amino acid transporter involved in glutamine-addicted cells, LAT1, was examined with the knockout cell lines. Once again, non-silencing and HIF-2α A and B knockout cells were plated at a density of 1 x 10⁵ per well of a six-well plate and incubated a total of 48 hours under either normoxic conditions, 6 hours hypoxia, 24 hours hypoxia or 48 hours hypoxia (Figure 16). The LAT1 antibody produced faint images, but no clear effect of HIF-2α knockout was observed in LAT1 expression.
Figure 16: LAT1 expression under increasing intervals of hypoxia. Western blot showing LAT1 expression in Huh-7 non-silencing (NS) and HIF-2α knockout lines (A & B) at different periods of hypoxia (1% O₂). (n=1)

The same time point of 48 hours which exhibited decreased ASCT2 expression was further examined with new LAT1 antibody using all knockout lines along with non-silencing and parent Huh-7 lines (Figure 17). All HIF-2α knockout lines appeared to show higher LAT1 protein expression levels compared to their non-silencing control, although the parent line also demonstrated expression comparable to the knockouts. LAT1 expression between normoxia and 48 hours of hypoxia did not appear to change appreciably. Expression in the parent and non-silencing control was more variable, but none of the cell lines showed a difference that was statistically significant.
Figure 17: LAT1 expression under long term hypoxia. Western blot showing Huh-7 parent (P), non-silencing (NS) and HIF-2α knockout lines (A, B, & C) under either 48 hrs. normoxia or hypoxia (1% O₂) (A). Densitometry showing relative LAT1 protein quantification (as a ratio of β-actin and normalized to NS normoxia) between cell lines. Differences between hypoxic LAT1 expression NS vs. A * (p = 0.0419), NS vs. B ns (p = 0.1044), NS vs. C ns (p = 0.2872) determined by one-way ANOVA (B). Continued on following page.
**Figure 17 continued.** Densitometry showing percent change in LAT1 expression between normoxic and hypoxic conditions in each cell line. NS vs. P ns (p = 0.9740), NS vs. A ns (p = 0.9941), NS vs. ns (p = 0.9813), NS vs. C ns (p = 0.9422) (C). (n=3), one-way ANOVA with Holm-Sidak’s test for multiple comparisons, +/- = SEM. Cutoff for p values (ns > 0.5), (* ≤ 0.05), (**) ≤ 0.01), (***) ≥ 0.001), (**** ≥ 0.0001).

qPCR was performed to examine mRNA expression of LAT1 during a 48-hour period of normoxia/hypoxia, repeating procedure used for ASCT2 (Figure 18). Average LAT1 mRNA expression was relatively consistent between cell line and oxygen level with the exception of the “C” HIF-2α knockout, which had a higher level of LAT1 mRNA under
hypoxia. The change in LAT1 mRNA between normoxia and hypoxia in each cell line showed variability between biological replicates but little net change overall; even the highest difference observed with the “C” HIF-2α knockout was not statistically significant compared to the non-silencing control.

Figure 18: LAT1 mRNA expression under long term hypoxia. qPCR showing LAT1 expression in Huh-7 parent line, non-silencing (NS) and HIF-2α knockout lines (A, B, & C) under normoxia vs. 48 hours hypoxia (1% O2). Differences between hypoxic LAT1 mRNA expression NS vs. A ns (p = 0.8921), NS vs. B ns (p = 0.9980), NS vs. C ** (p = 0.0062) determined by one-way ANOVA (A). Continued on following page.
Figure 18 continued. Percent change in LAT1 mRNA between normoxic and hypoxic conditions in each cell line. NS vs. P ns (p = 0.7039), NS vs. A ns (p = 0.9819), NS vs. B ns (p = 0.9911), NS vs. C ns (p = 0.2974) (B). (n=4), one-way ANOVA with Holm-Sidak’s test for multiple comparisons, +/- = SEM. Cutoff for p values (ns > 0.5), (* ≤ 0.05), (** ≤ 0.01), (*** ≥ 0.001), (**** ≥ 0.0001).
The HIF-2α overexpressing line was used, repeating the procedure for ASCT2, to examine LAT1 (Figure 19) under long-term hypoxia. Once again, the weak antibody signal makes the band identification difficult but both parent and overexpressing lines appear to show higher expression compared to the empty vector.

![Western blot showing LAT1 expression](image)

**Figure 19: LAT1 not induced under long-term hypoxia.** Western blot showing Huh-7 parent (P), empty vector pCDH (EV) and HIF-2α overexpressing (OE) cell lines under either 48 hrs normoxia or hypoxia (1% O₂). (n=1)

These results were compared to qPCR for LAT1 (Figure 20). The results do not show a statically significant difference compared to the empty vector control. Both cell lines show moderate induction of LAT1 under hypoxia, though less than the parent line.
Figure 20: HIF-2α overexpression failed to induce LAT1 mRNA expression. qPCR showing Huh-7 parent, empty vector pCDH and HIF-2α overexpressing under 48 hrs of normoxia or hypoxia (1% O₂). Statistical significance determined by t test between hypoxic ASCT2 expression in pCDH and overexpressing line (p = 0.39420). (n=1) +/- = SEM.

**Evaluation of xCT.** The amino acid transporter xCT, which plays a central role in glutathione homeostasis, was examined by western blot at multiple points of hypoxia with the non-silencing along with “A” and “B” HIF-2α knockout lines (Figure 21) to determine if xCT expression was influenced by HIF-2 under hypoxia. No differences in xCT expression were observed between cell lines under the intervals of hypoxia.
Evaluation of c-Myc. The transcription factor c-Myc known to regulate ASCT2 and LAT1 was examined by western blot with HIF-2α knockout lines and both parent as well as non-silencing controls at 48 hours of normoxia vs. hypoxia (Figure 22). Results show c-Myc expression is greatly reduced under long-term hypoxia but loss of HIF-2α does not appear to influence expression at either noromixa or hypoxia. The non-silencing and “B” knockout appear to have more abundant c-Myc protein bands under normoxia; however, this appears to be due to overloaded lanes evident by the β-actin loading control.
Figure 22: c-Myc shows strong repression under long-term hypoxia. Western blot showing Huh-7 parent (P), non-silencing (NS) and HIF-2α knockout lines (A, B, & C) under either 48 hrs normoxia or hypoxia (1% O₂) (A). Densitometry showing c-Myc protein expression adjusted to β-actin loading control (B). (n=1)
The experiment was repeated using the parent, empty vector and HIF-2α overexpressing lines (Figure 23). Expression of c-Myc was consistent across cell lines under normoxia and repressed overall under 48 hours of hypoxia without any visible difference in the HIF-2α overexpressing line.

**Figure 23: c-Myc was not induced under long-term hypoxia.** Western blot showing Huh-7 parent (P), empty vector pCDH (EV) and HIF-2α overexpressing (OE) cell lines under either 48 hrs normoxia or hypoxia (1% O₂) (A). Continued on following page.
**Cell Proliferation.** To examine the possible effect of HIF-2 loss on cell proliferation under hypoxia, Huh-7 parent, non-silencing and HIF-2α knockout lines were plated at a density of $2 \times 10^4$ cells in triplicate wells of a 24-well plate and incubated under either 48 hours of hypoxia or normoxia. Following incubation, cells were trypsinized and resuspended in 100 μL of PBS. Cells were counted using a hemacytometer and the average of all three replicates were taken (Figure 24). While the parent line had a lower cell count under hypoxia, results show no difference in cell growth in HIF-2α knockout lines or the non-silencing control between normoxic and long-term hypoxic conditions.
Figure 24: Growth rate of HIF2α knockout lines was not impaired under hypoxia. Direct cell count of Huh-7 parent, non-silencing (NS), and HIF-2α knockout lines (A, B & C) under either 48 hours normoxia or hypoxia (1% O₂). (n=1) +/- = SEM.
DISCUSSION

This project successfully established three independent Huh-7 cell lines lacking HIF-2α expression as well as one line expressing GFP-tagged HIF-2α, providing models to study the role of HIF-2α in human hepatocellular carcinoma. Generation of these lines highlights several observations regarding HIF-2α in the Huh-7 cell line. First, cells appear to exhibit a low level of stabilized HIF-2α under standard cell culture conditions. The most likely explanation for this is the low oxygen permeability of a liquid medium used in cell culture. While certainly below the 18-20% oxygen tension within a cell culture incubator, it is difficult to estimate the degree of hypoxia in vitro that cells experience. HIF-2α can begin accumulating at oxygen levels as high as 5% and comparison between protein bands suggests normoxic cell culture conditions are not within extreme ranges of 1% oxygen. Another possible explanation is HIF-2α may be upregulated in the Huh-7 phenotype due to a non-oxygen-related mechanism such as dysregulation of the degradation components including pVHL or PHDs.

Results from transfection with an expression vector show forced HIF-2α expression does not accumulate beyond endogenous HIF-2α expression at normoxic cell culture conditions. This demonstrates a very rapid and efficient degradation pathway suppressing HIF-2α accumulation. Inhibition of this pathway is still necessary to see any appreciable HIF-2α protein from the overexpressing cell line. After only 6 hours of
1% O₂, the GFP-tagged HIF-2α accumulation exceeds the endogenous HIF-2α under the same conditions. This data is supported by the fluorescent microscopy which shows a small amount of cellular GTP-tagged HIF-2α present under normoxia, all of which exists outside the nucleus. After 6 hours of 1% O₂, increased accumulation is evident as well as nuclear localization in some cells. Whole-cell fluorescence appears to peak or remain steady between 24 and 48 hours of hypoxia. Cobalt chloride used as a mimic of hypoxia showed surprisingly more effective results compared to actual hypoxia. A low dose of 50 μM displayed more nuclear HIF-2α than any time point of 1% O₂. However, while it may stabilize HIF protein and promote translocation to the nucleus in liver cancer cells, Befani et al. (2013) have shown cobalt chloride does not necessarily result in transcriptionally active HIF protein, making it less suitable to study downstream targets.

Perhaps the strongest finding from this study is the relationship between HIF-2 and ASCT2. Protein data showed ASCT2 expression is maintained at levels near those of normoxia in control cell lines while the loss of HIF-2 resulted in a decrease in ASCT2 over the course of long-term hypoxia, ranging between 25-50% across the knockout lines by 48 hours. qPCR data showed an induction of ASCT2 mRNA under long-term hypoxia in the control lines. This induction was not only absent in all three knockout lines, but each showed a decrease in ASCT2 mRNA, most notably knockout “B”. As the increase in ASCT2 mRNA did not appear to lead to an increase in protein, it is possible this transcriptional induction is necessary simply to maintain normal levels of ASCT2. Comparing the mRNA data to the protein data, a pattern is evident where the higher the
induction of ASCT2 mRNA under hypoxia, the closer ASCT2 protein was maintained to its normoxic expression level. A similar pattern was also seen among the knockout lines, where a larger decrease in ASCT2 mRNA appeared to correlate to lower ASCT2 protein, with the exception of the more variable mRNA expression of the “C” HIF-2α knockout.

One possible explanation for this relationship involves c-Myc, of which ASCT2 is a transcriptional target. HIF-1 inhibits c-Myc by enhancing MXI1 and interfering with Myc/Max association while HIF-2 enhances c-Myc by promoting the interaction between Myc/Max (Elorza et al., 2012). Under hypoxia, these competitive interactions between HIFs may be responsible for the observed ASCT2 expression pattern. By eliminating HIF-2α, the balance may be tipped in favor of an overall inhibitory effect on c-Myc target genes, explaining the decrease in ASCT2 observed in the knockout lines (Figure 25). The protein expression of c-Myc was observed in the same 48 hour normoxia vs. hypoxia window by western blot. Results showed a large repression of c-Myc after 48 hours across all cell lines with no differences in the HIF-2α knockout lines. This suggests HIF-2 does not influence the expression of c-Myc and may only modulate its activity by promoting Myc/Max complex formation, though this function may be more crucial under hypoxia where Myc expression is reduced. Another possibility is that ASCT2 may be a direct target gene of HIF-2, although to date, this has yet to be demonstrated.
Figure 25: Possible model of ASCT2 regulation by HIF. HIF-2 may promote ASCT2 expression via Myc and/or as a direct transcription target gene. HIF-1 and HIF-2 are known to have opposing effects on Myc/Max assembly. Loss of HIF-2 may tip the balance towards decreased expression.

If loss of HIF-2 suppresses ASCT2 expression under hypoxia, the overexpression of HIF-2 might be expected to enhance ASCT2. However, this was not the observed result when overexpressing the HIF-2α subunit. Slightly higher ASCT2 protein expression was observed under normoxia but no difference was seen under 48 hours of hypoxia. At the transcriptional level, ASCT2 mRNA did not exceed that of the parent or empty vector control. One possible reason is that transcriptionally active HIF-2, as a heterodimer, could be limited by the expression of its β subunit. Another, more likely explanation is that the large C-terminal GFP tag on the HIF-2α fusion protein interferes with its function as an activating transcription factor.
While the link between ASCT and HIF-2 appears to be supported at both the transcriptional and protein levels, LAT1 shows a much less clear relationship. Also, issues with the LAT1 antibody resulted in long exposures and heavy background noise during imaging, making accurate densitometry analysis difficult in some cases. Based on protein expression, LAT1 appears to be expressed higher in all three knockout lines under normal conditions when compared to their non-silencing control. When compared to the Huh-7 parent line, however, this pattern appears modest. It is difficult to determine if loss of HIF-2 actually influences the resting expression of LAT1 considering this discrepancy between controls. Off-target effects and antibiotic selection pressure are variables that could potentially alter gene expression in the CRISPR lines, including the non-silencing control, and complicate comparison to the parent line. While variation existed between cell lines in LAT1 expression, there appeared to be very little difference between normoxia and hypoxia, suggesting chronic low oxygen (48 hours) does not influence LAT1 with or without HIF-2 expression. Transcriptional data largely supported this pattern, showing little difference between normoxic and hypoxic LAT1 mRNA. The exception was the HIF-2α knockout “C” line, showing a large increase under hypoxia, though the difference was not statistically significant compared to the difference in the non-silencing control.

Despite also being a target of c-Myc, the protein expression differs greatly from ASCT2. It is possible another mechanism sustains LAT1 expression, but not ASCT2, under hypoxia that is independent of HIF-2. It is unusual, however, that no effect was
seen on LAT1 expression in response to loss of HIF-2α as other studies offered evidence that LAT1 can be regulated by HIF-2, including as a direct target gene (Elorza et al., 2012). One possibility is that the LAT1 promoter containing HRE sequences may allow it to be a target for HIF-1 as well as HIF-2 and therefore may not be affected by the loss of one HIF as ASCT2 is. While knockdown of HIF-2α has been shown to decrease LAT1 expression in renal cell carcinoma, the complexities of the hypoxic response including possible feedback between HIF isoforms or tissue-specific roles may account for differences in other cancer models. Generation of similar knockouts in other hepatocellular carcinoma lines such as SK-HEP or Hep G2 would be one potential next step for further evaluating the relationship. Examining the overexpression of HIF-2 in Huh-7 cells could also support whether or not there is an effect on LAT1 in HCC. The data obtained from the HIF-2α overexpressing line showed no change in LAT1 expression compared to controls at either the transcriptional or protein level. However, this data cannot be accurately evaluated until the transcriptional functionality of the HIF-2α-GFP fusion protein can be validated.

The amino acid transporter xCT was also examined as another possible candidate for regulation by HIF-2 under hypoxia. xCT exchanges glutamate for cystine, which is required for synthesis of glutathione as a means of neutralizing ROS. HIF-1 has been shown to regulate xCT, but it has not been shown to be a target of HIF-2. Results across multiple time points of hypoxia do not show changes in xCT expression with loss of HIF-2α, suggesting HIF-2 is not involved in regulating xCT under hypoxia.
The establishment of HIF-2α knockout lines demonstrates that loss of HIF-2 does not appear to induce cell death or inhibit proliferation in vitro even under hypoxia as long as 48 hours. In order to compare cell proliferation under hypoxia, several methods were considered. MTT and XTT assays are frequently used, but these are based on a metabolic reaction occurring within the mitochondria. As both HIF-1 and HIF-2 alter and suppress mitochondrial function, hypoxia may alter the accuracy of these assays. A simple, direct cell count was performed to observe any difference between normoxic and hypoxic cell growth for each cell line. Due to the variability in initial cell counts used to plate cell lines, comparing growth between cell lines would not be considered accurate due to the high chance of error. However, the normoxic and hypoxic groups within each cell line can be directly compared as they were plated using the same initial concentration of cells. No cell line shows a statistically significant difference between normoxia and hypoxia in terms of number of cells. As cells growing in monolayer with nutrient-rich medium do not replicate the stresses, complexity and interactions of a 3D tumor microenvironment, the cell lines would need to be evaluated in vivo to evaluate tumorigenicity.

This study successfully generated human hepatocellular carcinoma cell lines lacking HIF-2α and highlights a novel mechanism where HIF-2 appears to maintain amino acid transporter ASCT2 under long-term hypoxia. This data suggests future directions to further investigate the role of HIF-2 on glutamine metabolism under hypoxia, including examining amino acid trafficking and expression of key enzymes such as glutaminase. A pilot study using a xenograft model with one HIF-2α knockout line is
currently underway to examine the *in vivo* effect with loss of HIF-2 on tumor
development. The potential role of HIF-2 in oncogenesis and regulation of key survival
pathways makes it an attractive target for cancer treatment strategies


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