RESEARCH ARTICLE

Glucose and glutamine availability regulate HepG2 transcriptional responses to low oxygen [version 1; referees: 2 approved, 1 approved with reservations]

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Abstract

Background: Little is known about the impact of nutrients on cellular transcriptional responses, especially in face of environmental stressors such as oxygen deprivation. Hypoxia-inducible factors (HIF) coordinate the expression of genes essential for adaptation to oxygen-deprived environments. A second family of oxygen-sensing genes known as the alpha-ketoglutarate-dependent dioxygenases are also implicated in oxygen homeostasis and epigenetic regulation. The relationship between nutritional status and cellular response to hypoxia is understudied. *In vitro* cell culture systems frequently propagate cells in media that contains excess nutrients, and this may directly influence transcriptional response in hypoxia.
Methods: We studied the effect of glucose and glutamine concentration on HepG2 hepatoma transcriptional response to low oxygen and expression of hypoxia inducible factor-1α (HIF-1α). Mass spectrometry confirmed low oxygen perturbation of dioxygenase transcripts resulted in changes in DNA methylation.
Results: Under normoxic conditions, we observed a significant upregulation of both HIF-target genes and oxygen-dependent dioxygenases in HepG2 cells cultured with physiological levels of glucose or glutamine relative to regular DMEM media, demonstrating that excess glutamine/glucose can mask changes in gene expression. Under hypoxic conditions, *CA9* was the most upregulated gene in physiological glutamine media while *TETs* and *FTO* dioxygenases were downregulated in physiological glucose. Hypoxic regulation of these transcripts did not associate with changes in HIF-1α protein expression. Downregulation of *TETs* suggests a potential for epigenetic modulation. Mass-spectrometry quantification of modified DNA bases confirmed our transcript data. Hypoxia resulted in decreased DNA hydroxymethylation, which correlated with *TETs* downregulation. Additionally, we observed that *TET2* expression was significantly downregulated in patients with hepatocellular carcinoma, suggesting that tumour hypoxia may deregulate *TET2* expression resulting in global changes in DNA hydroxymethylation.
Conclusion: Given the dramatic effects of nutrient availability on gene

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Referee Status: ✓ ✓ ?

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expression, future *in vitro* experiments should be aware of how excess levels of glutamine and glucose may perturb transcriptional responses.

Keywords
Glucose, glutamine, HepG2, hypoxia, HIF, dioxygenases, TET, methylation, hepatocellular carcinoma

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Introduction

In most organisms, energy usage is tightly regulated by tissue sensing of nutritional status and the coordination of cellular responses. Oxygen is an essential component in most physiological functions and nutrient availability may influence how cells respond to environments where oxygen is limited. Hypoxia inducible factors (HIFs) are a master regulator of oxygen sensing and define cellular transcriptional response in low oxygen. HIFs activate genes that are essential for cells to survive under low oxygen conditions which helps cells reduce energy demands by limiting oxygen consumption, for instance employing glycolysis in place of oxidative phosphorylation (Figure 1)[1]. Another gene family known as the alpha-ketoglutarate-dependent dioxygenases also regulate metabolic homeostasis under low oxygen. They catalyse a range of oxidative processes including hydroxylation and have an absolute requirement for molecular oxygen[2]. TET dioxygenases are sensitive to changes in oxygen levels and their hydroxylase activities are significantly altered in solid tumours that are characteristically hypoxic[3]. TET mediates DNA demethylation through the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-carboxylcytosine (5caC) and 5-formylcytosine (5fC), subsequently followed by base excision repair (Figure 1)[4]. Hypoxia-driven changes in TET activity can result in epigenetic alterations that contribute to cancer progression[1].

Hotamisligil *et al*. hypothesized that metabolic systems evolved at a time when modern day pressures such as nutrient surplus were absent or uncommon[5]. As a consequence, cellular exposure to nutrient excess may alter the magnitude of biological responses and modify metabolic homeostasis, innate immune sensing and inflammatory responses[6,7]. Hence, we predict that cellular transcriptional response in hypoxia will be linked to nutrient availability. There is a significant body of literature on the importance of glucose and glutamine concentrations for *in vitro* cell proliferation[8–15]. However, relatively little is known about the effect of these nutrients on hypoxia-driven transcriptional responses and epigenetic changes.

The HepG2 human hepatoma cell line is one of the most widely characterized cells in terms of its signalling pathways and transcriptional responses with a large collection of high-throughput functional genomic datasets available in repositories such as Gene Expression Omnibus, ArrayExpress and ENCODE, containing over 1000, 300 and 800 HepG2-based datasets, respectively. However, datasets describing the effect(s) of nutrients on cellular transcriptome have remained scant. Given the widespread use of RNA-sequencing technologies and the current drive to single cell approaches for transcriptional profiling and bio-marker research, the media requirements for any *ex vivo* cell studies are important to consider and standardize. We assessed the effects of media on the transcriptional response of selected HIF-target genes along with oxygen-dependent dioxygenases[2]. Alterations in DNA methylation levels as a result of oxygen deprivation were also assessed in our preferred media choice.

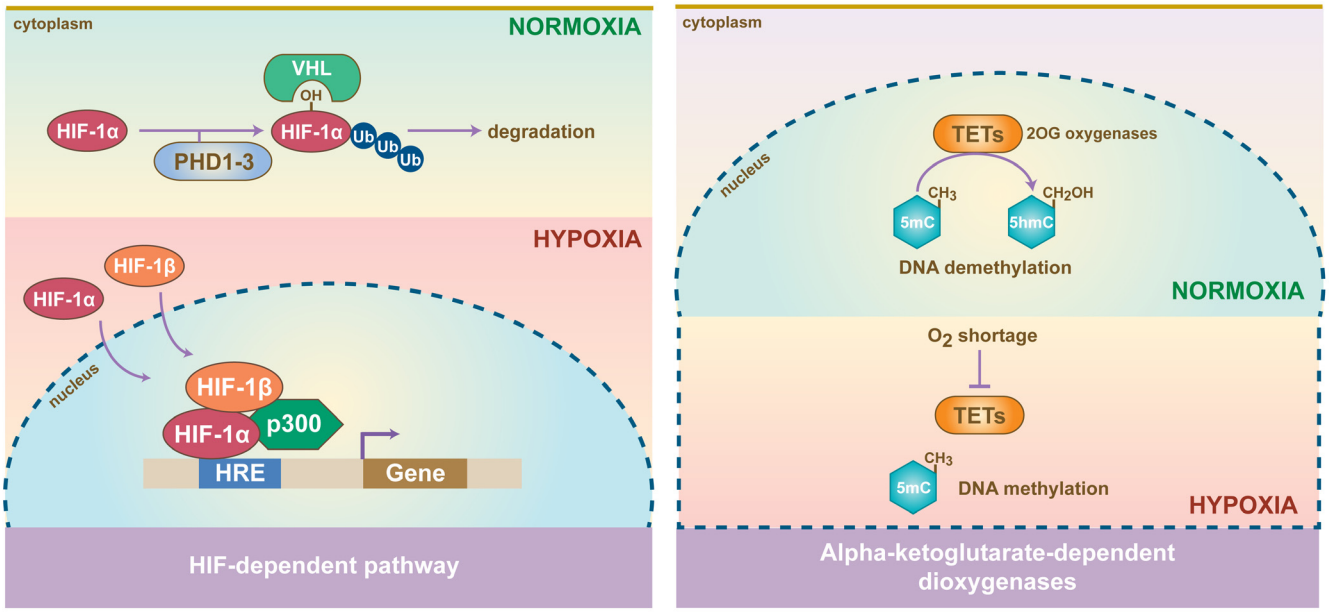

Figure 1. Nutrient availability affects cellular response to stress signals. Cellular response to low oxygen as mediated through HIFs and alpha-ketoglutarate-dependent dioxygenases signalling. HIF-1α is hydroxylated by prolyl-4-hydroxylases 1-3 (PHD1-3) under normoxic conditions and targets the protein for proteasomal degradation by the von Hippel-Lindau (VHL)-E3 ubiquitin ligase complex. Under hypoxia, PHDs are no longer active, allowing s HIF-1α translocation to the nucleus where it heterodimerises with HIF-1β. This heterodimer recognizes and binds to the hypoxia responsive element (HRE), recruits p300 and activates its target genes. The HIF-independent pathway involves the oxygen-dependent dioxygenases. TETs promote the conversion of 5-methylcytosine to 5-hydroxymethylcytosine to achieve demethylation. Under hypoxia, it is thought that TETs promote DNA hypermethylation through a decrease in its activity due to oxygen shortage, resulting in the accumulation of 5-methylcytosine and DNA hypermethylation in tumours.

Results and discussion

Dulbecco's Modified Eagle's medium (DMEM) is one of the most widely used media for culturing cells *in vitro* and contains 25mM glucose and 2mM Glutamax (L-alanyl-L-glutamine), representing 4-5-fold higher concentrations than normal physiological levels. We studied the effect of reducing glucose and glutamine levels to 5mM and 0.5mM respectively on cellular transcription[12–17]. HepG2 cells proliferated in media with reduced glucose or glutamine at a comparable rate to those in DMEM and no adverse effects on cell viability were noted.

Glucose and glutamine concentrations modulate transcript levels of selected HIF-target and dioxygenase genes

Hypoxia and DNA hypermethylation are common in solid tumours, where reduced oxygen levels activate the HIF transcriptional complex[18,19], orchestrating the expression of a wide range of genes in an attempt to restore metabolic homeostasis[20,21]. A recent study showed the reduced TET enzymatic activity in hypoxic tumour cells leads to the accumulation of 5-methylcytosine and cancer progression[3].

We investigated the effect of culturing HepG2 cells in media with different glucose and glutamine concentrations on transcript levels of several HIF-target genes (*CA9, GLUT1, SCD1*, *BNIP3* and *VEGFA*) and dioxygenases (*TETs*, *ALKBH5* and *FTO*) genes under normoxic (20% oxygen) and hypoxic (1% oxygen) conditions. Under normoxic conditions, culturing cells in physiological glutamine or glucose media resulted in a significant change in gene transcript levels compared to cells propagated in DMEM (Figure 2A). For example, we noted increased mRNA levels of *GLUT1, SCD1, VEGFA, TET2*, and *TET3* in cells cultured in physiological glutamine or glucose compared to DMEM, suggesting that excess nutrients may dampen transcriptional activity. Comparing gene expression data from HepG2 cells cultured in low glucose or glutamine media relative to DMEM showed significant effects on gene expression and highlighted the gene-specific nature of the response (Figure 2B).

Exposure of HepG2 cells cultured in DMEM to low oxygen increased the transcription of several HIF-target genes (*CA9,*

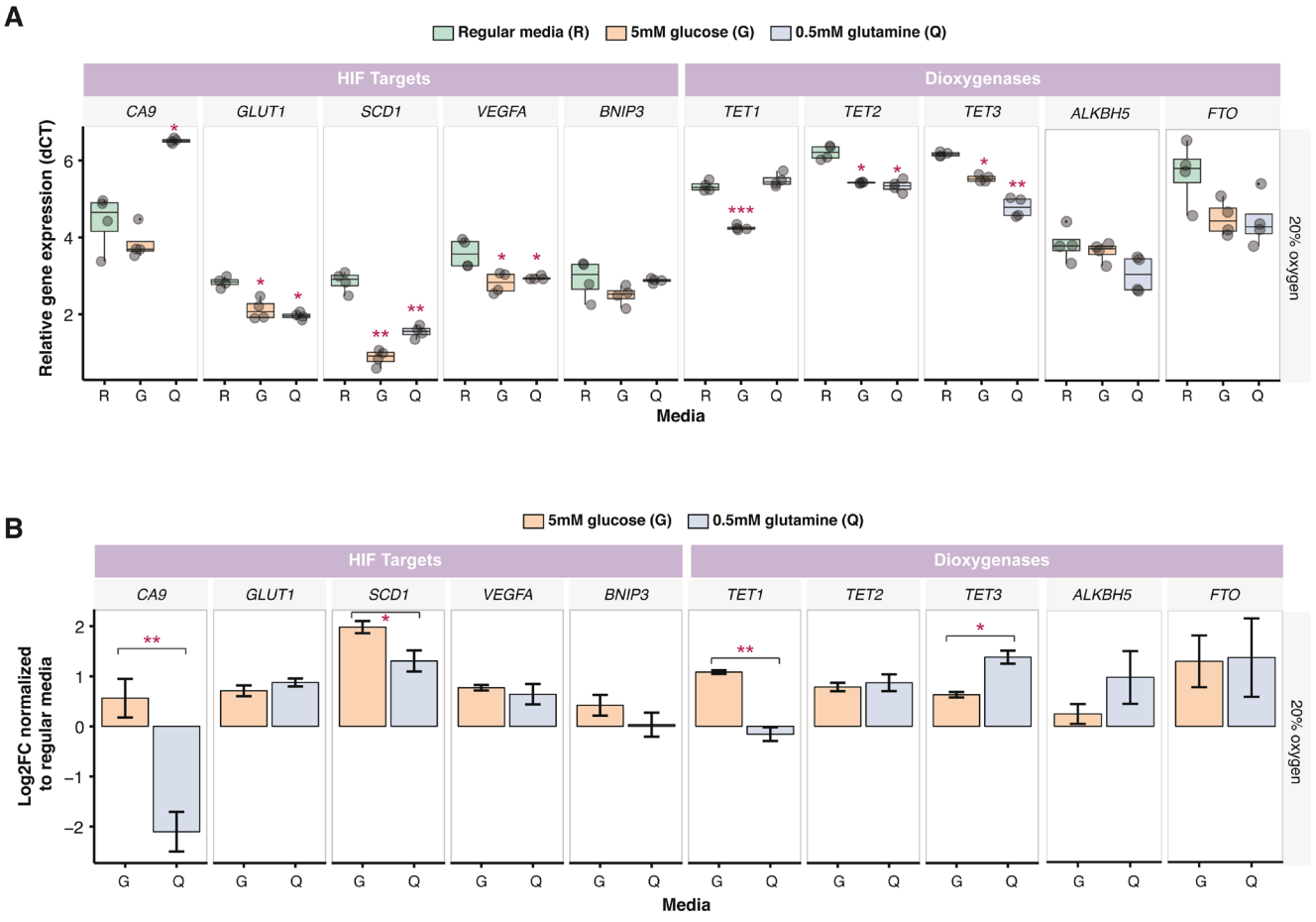

Figure 2. Glucose and glutamine availability affect HepG2 transcript levels under ambient culture conditions. (**A**) Transcript levels of selected HIF-target genes (*CA9, GLUT1, SCD1, BNIP3, VEGFA)* and dioxygenases (*TET1, TET2, TET3, ALKBH5* and *FTO*) in HepG2-NTCP cells cultured under ambient oxygen conditions (20%). Data is expressed as normalized values (ΔCt) relative to the internal housekeeping control *Beta-2-Microglobulin (B2M)* (n = 4; error bars ± SEM). (**B**) Fold induction represented as log2 fold change after normalization of physiological glutamine or glucose values relative to DMEM (n = 4; error bars ± SEM). A Kruskal-Wallis ANOVA with Bonferroni correction was used, p-values were indicated by the following: * < 0.05, ** < 0.01, *** < 0.001. R = regular media, G = physiological glucose, Q = physiological glutamine.

SCD1, *BNIP3* and *VEGFA*) and the magnitude of the "hypoxic response" was dependent on the culture media in a gene-specific fashion (Figure 3A). For example, hypoxic induction of *CA9* was two-fold higher in HepG2 cells cultured in 0.5mM glutamine compared to DMEM, whilst mRNA levels for the *TETs* and *FTO* were significantly reduced in 5mM glucose but not in DMEM (Figure 3A). This observation is consistent with a recent study reporting reduced TET activity in hypoxic tumour samples that associated with reduced 5hmC measurements in solid tumours[3]. Taken together, it is likely that excess glutamine/glucose levels perturb the metabolic cues that regulate cellular transcription and cellular responses to stress signals. The breakdown of homeostasis due to nutrient overload leading to aberrant cellular signalling is widely acknowledged to drive a myriad of pathologies such as cancer[6,22], fatty liver disease[23,24] and type 2 diabetes[25–27].

Next, we determined whether the effect(s) of glucose/glutamine levels on HIF-target gene expression was explained by changes in HIF protein expression. HepG2 cells express HIF-1α under low oxygen conditions in all three media types (Figure 3B), with an approximate 50% reduction in expression in cells cultured in 5mM glucose media (Figure 3B). In HepG2 cells stably expressing a luciferase reporter under the control of a hypoxia responsive (HRE) promoter, we observed a significant reduction in promoter activity in hypoxic cells cultured in 5mM glucose media (Figure 3C), consistent with reduced HIF-1α expression. These observations support an association between HIF-1α expression and HRE reporter activity, however, the impact of low glucose or glutamine on HRE activity did not correlate with other host transcriptional responses, demonstrating the simplistic nature of the reporter system. This illustrates the complex nature of HIF interactions with host gene promoter or enhancer elements, where other factors, such as the chromatin and methylation landscape determine the accessibility of any particular region to HIFs.

Hypoxia regulates epigenetic changes

We predict that reduced *TET* expression in hypoxia would result in decreased DNA hydroxymethylation and increased DNA methylation. Since we observed a robust downregulation of TETs when HepG2 cells were cultured in 5mM glucose

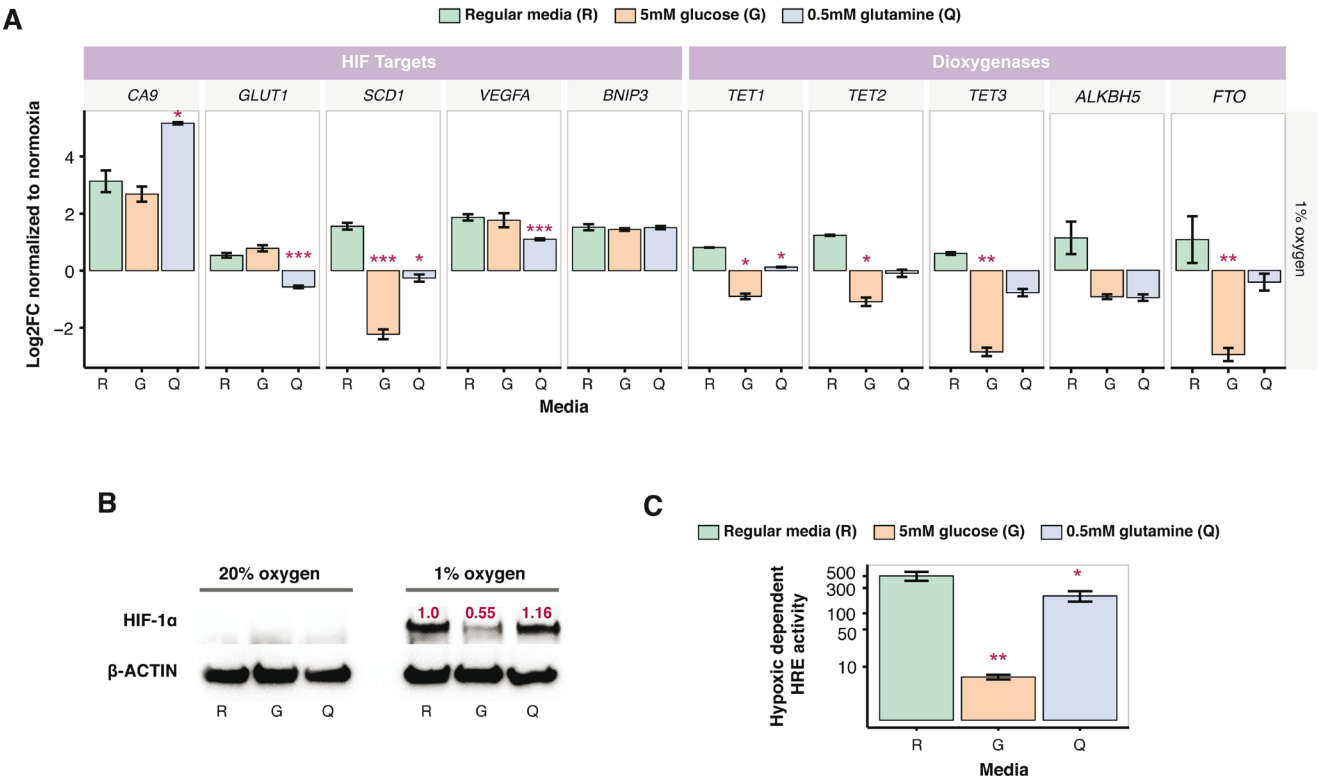

Figure 3. Glucose and glutamine availability affect HepG2 transcript levels under low oxygen. (**A**) Transcript levels of known HIF-target genes (*CA9, GLUT1, SCD1, BNIP3, VEGFA)* and dioxygenases (*TET1, TET2, TET3, ALKBH5* and *FTO*) were determined in HepG2-NTCP cells cultured under 1% oxygen for 48h. Hypoxic expression levels were expressed relative to normoxic values (n = 2–4; error bars ± SEM). (**B**) HIF-1α expression in HepG2-NTCP cells cultured in 20% and 1% oxygen for 48h. Densitometric quantification of HIF-1α expression after normalizing relative to B-actin levels are depicted in red font. (**C**) HepG2 cells expressing a reporter plasmid encoding a hypoxic responsive element (HRE) driving luciferase expression were cultured under 20% and 1% oxygen for 48h. Luciferase levels under hypoxic conditions are expressed relative to normoxic values (n = 16–24; error bars ± SEM). A Kruskal-Wallis ANOVA with Bonferroni correction was used, where p-values were indicated by the following: * < 0.05, ** < 0.01, *** < 0.001. R = regular media, G = physiological glucose, Q = physiological glutamine.

(Figure 3A), we selected this media of choice for subsequent mass-spectrometry quantification of 5mC and 5hmC. Hypoxic cells showed a modest increase in DNA methylation and a significant reduction in DNA hydroxymethylation (Figure 4). Low oxygen-dependent reduction of DNA hydroxymethylation is concomitant with a reduction in *TET* gene expression as they catalyse the conversion of 5mC to 5hmC (Figure 3A). This observation is consistent with another report by Thienpont *et al.*[3], suggesting that hypoxia-signalling promotes aberrant DNA methylation and can alter the epigenetic landscape of hepatocyte-specific transcription factors. Of note a recent study reported that low glucose could stabilize *TET2*[28]. Hence, glucose surplus in regular DMEM could perturb *TET2* expression and may mask any further reduction of 5hmC in hypoxia.

TET2 expression levels were decreased in hepatocellular carcinoma

Previous studies have demonstrated that oxidative derivatives of 5mC were decreased in hepatocellular carcinoma (HCC)[29]. This correlated with a significant decrease in *TET2* expression levels and alpha-ketoglutarate content in patients with HCC, suggesting that decrease in TET enzymatic activity resulted in altered expression of DNA methylation enzymes[29,30]. Independently, using HCC mRNA expression profiles from The Cancer Genome Atlas, we found reduced *TET2* transcripts in tumour compared to non-tumour samples (Figure 5). However, *TET1* and *TET3* expression levels were upregulated in tumour samples, suggesting that they may compensate for reduced *TET2* levels in HCC (Figure 5). Importantly, decreased *TET2* expression in HCC confirmed our earlier observation on hypoxic treated HepG2 cells cultured in physiological glucose media (Figure 3A and Figure 4).

Conclusion

We found that a simple change in cell culture media resulted in significant changes in hypoxia transcriptional response (Figure 2 and Figure 3), occurring through both HIF-dependent and independent pathways. We predict that these changes in mRNA levels will be reflected in protein translation. Moreover, there appears to be global alterations in hepatocellular DNA methylation in hypoxia conditions (Figure 4) and reduced *TET2* transcript levels in HCC tumours (Figure 5). We suggest that *in vitro* studies measuring cellular transcriptional responses consider the use of defined media to ensure robust datasets and to promote the translation of *in vitro* datasets to *in vivo* physiological events.

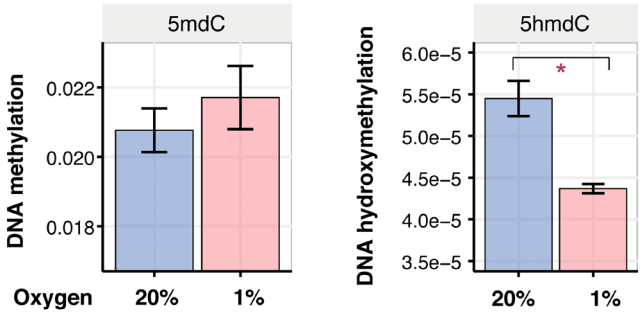

Figure 4. Hypoxia alters HepG2 DNA methylation. Mass spectrometry quantification of DNA methylation (5-methyl-deoxycytidine/5mdC) and DNA hydroxymethylation (5-hydroxymethyl-2'-deoxycytidine / 5hmdC) in HepG2 cells cultured in physiological glucose media at 20% and 1% oxygen for 48 hours. A student T-test comparison was used, p-values were indicated by the following: * < 0.05.

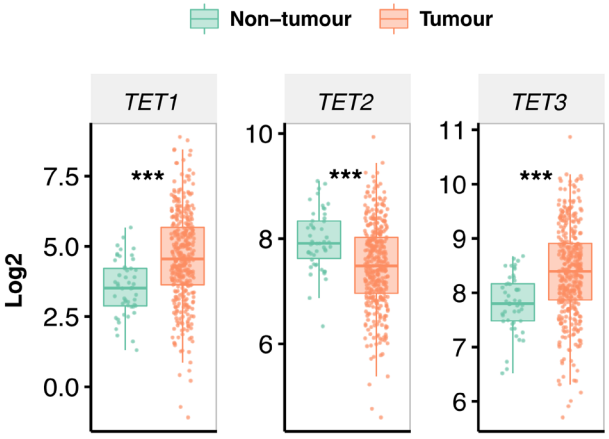

Figure 5. Expression distribution of *TETs* mRNA in hepatocellular carcinoma. Nonparametric Mann-Whitney-Wilcoxon test was used to compare the distribution of *TETs* expression in tumour and adjacent non-tumour samples. Asterisks *** represent P < 0.0001.

Methods

Cell culture

Human hepatoma HepG2 cells were cultured with regular, physiological glucose or glutamine media as follows: regular media is basic DMEM (ThermoFisher Scientific - A1443001) supplemented with 25mM glucose, 2mM glutamax, 10% fetal calf serum (FCS) and 1x penicillin-streptomycin (P/S); physiological glucose is basic DMEM with 5mM glucose, 2mM glutamax, 10% FCS and 1x P/S; physiological glutamine media is basic DMEM with 25mM glucose, 0.5mM glutamax, 10% FCS and 1X P/S. Cells were seeded at 26,000 cells/cm^2 in collagen (Sigma) coated 6-well plates and incubated for 48h under 20% or 1% oxygen (Invivo2, Baker Ruskinn).

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Cellular RNA samples were extracted from normoxic and hypoxic samples using the RNeasy Mini kit (Qiagen) with in-column DNAse digestion performed according to the manufacturer's instructions. One-step qPCRs for *CA9*, *GLUT1*, *BNIP3* and *VEGFA* were performed using a TaqMan master mix (Takyon, Eurogentec) and TaqMan MGB probes (Applied Biosystems). For detection of *SCD1*, *TET1*, *TET2*, *TET3, FTO* and *ALKBH5* mRNA, cDNA syntheses from 1µg of total RNA were performed using a QuantiTect Reverse Transcription Kit (Qiagen), followed by qPCR using a SYBR green master mix (Applied Biosystems) (Table S1). The hypoxia insensitive housekeeping gene *Beta-2-Microglobulin* (*B2M*) was used as

an endogenous control. Log2 fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. In order to depict gene expression levels under normoxia, ΔCt values (Ct gene – Ct *B2M*) were calculated.

HIF-1α western blotting

HepG2-NTCP cells were seeded at 26,000 cells/cm^2 in collagen (Sigma) coated 6-well plates in the various media and left to adhere overnight. Cells were incubated at 20% or 1% oxygen for 48h and lysed using 8M urea lysis buffer. Approximately 20µg of protein was separated by PAGE and transferred to Polyvinylidene difluoride (PVDF) membrane and incubated with primary antibodies for HIF-1α (BD Biosciences, USA), or β-actin (Thermo Fisher Scientific, USA). Bound antibodies were detected using horseradish peroxidase (HRP) conjugated secondary antibodies (Dako, Agilent Technologies, USA) with chemiluminescent detection (SuperSignal, Thermo Fisher Scientific) using a PXi Touch Gel Imaging System (Syngene).

HRE reporter assay

HepG2-NTCP cells cultured as described above were lysed and luciferase activity assessed using GloMax luminometer in accordance with the manufacturer's instructions (Promega). Lysates were quantified for protein concentration using a BCA Protein Assay Kit (Thermo Fisher Scientific) and luciferase activity (relative light units, RLU) expressed relative to protein concentration.

DNA digestion and HPLC-MS/MS analysis

DNA samples were incubated in hydrolysis solution containing 45 mM NaCl (Invitrogen), 9 mM $MgCl_2$ (Ambion), 9 mM Tris-HCl (pH 7.9, Gibco), 25 U Benzonase Nuclease (Sigma-Aldrich), 5 mU Phosphodiesterase I (Sigma-Aldrich), 0.5 µg Alkaline phosphatase (Sigma-Aldrich), 9.36 ng/µL EHNA hydrochloride (Sigma-Aldrich) and 3.52 µM Deferoxamine (Sigma-Aldrich) at 37°C for 4 hours. The digested samples were filtered with Amicon Ultra-0.5 mL 10K centrifugal filters (Merck Millipore) to remove the proteins. The same volume of HPLC buffer A (H_2O containing 10 mM ammonium acetate, pH 6.0) was added to the filtered samples, and then subjected to HPLC-MS/MS analysis.

The HPLC-MS/MS analysis was carried out with 1290 Infinity LC Systems (Agilent) coupled with a 6495B Triple Quadrupole Mass Spectrometer (Agilent). A ZORBAX Eclipse Plus C18 column (2.1 x 150mm, 1.8-Micron, Agilent) was used. The column temperature was maintained at 40°C, and the solvent system was water containing 10mM ammonium acetate (pH 6.0, solvent A) and water-acetonitrile (60/40, v/v, solvent B) with a 0.4 mL/min flow rate. The gradient was: 0–5 min; 0 solvent B; 5–8 min; 0–5.63 % solvent B; 8–9 min; 5.63% solvent B; 9–16 min; 5.63–13.66% solvent B; 16–17 min; 13.66–100% solvent B; 17–21 min; 100% solvent B; 21–24.3 min; 100–0% solvent B; 24.3–25 min; 0% solvent B. The dynamic multiple reaction monitoring mode (dMRM) of the MS was used for quantification. The source-dependent parameters were as follows: gas temperature 230°C, gas flow 14 L/min, nebulizer 40 psi, sheath gas temperature 400°C, sheath gas flow 11 L/min, capillary voltage 1500 V in the positive ion mode, nozzle voltage 0 V, high pressure RF 110 V and low-pressure RF 80 V, both in the positive ion mode. The fragmentor voltage was 380 V for all compounds, while other compound-dependent parameters were summarized in Table S2.

HCC RNA-sequencing cohort

The liver cancer dataset (LIHC) used in this study was obtained from The Cancer Genome Atlas (TCGA)[31]. Gene expression profiles for LIHC were downloaded from the Broad Institute GDAC Firehose (http://gdac.broadinstitute.org/). RSEM normalized expression profiles were converted to $\log_2(x + 1)$ scale and separated according to TCGA barcodes to tumour and non-tumour categories. Box plots were generated using the R ggplot2.

Statistical analysis

Statistical analyses were performed using R (version 3.5.1). Details of statistical tests performed can be found in the figure legends.

Data availability

Raw data for the study, including values for mRNA levels, mass spectrometry measurements, luciferase measurements and raw western blot images are available on OSF: https://doi.org/10.17605/OSF.IO/RXKPW[32].

Grant information

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Supplementary material

Tables S1 and S2. Table S1: List of primer sequences used for qPCR analyses; Table S2: Compound-dependent LC-MS/MS parameters used for nucleosides quantification.

Click here to access the data

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Version 1

Referee Report 16 October 2018

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? **Daniel A. Tennant**
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The manusript from Lai *et al.* describes an investigation into the role of the supraphysiological levels of nutrients used in conventional tissue culture on *in vitro* investigations into aspects of dioxygenase function and hypoxia-inducible factor (HIF) transcription factor signalling. Overall it is a well-written and there is a clear narrative throughout. The experiments are performed with excellent reproducibility, resulting in data that are easy to interpret. However, there are some over-simplifications in the text and data analysis that result in a degree of over-interpretation at times that could do with resolving.

Specific points

1. The authors use Glutamax throughout as their means of supplementing glutamine; an alanyl-glutamine dipeptide. It would be beneficial if they could tighten their text to reflect that they are supplementing both amino acids to the medium rather than just glutamine. This may be important as, although not investigated, this additional nutrient may contribute to the phenotype.
2. A further point throughout is that physiologically-relevant concentrations of glucose or Glutamax are supplemented, compared to supraphysiological concentrations of both. Although it is important to observe the effects of reducing each nutrient separately as part of an experimental system, the more relevant condition in physiological terms is the simultaneous reduction in both nutrients. Given that physiological concentrations of glucose and glutamax were shown by the authors to have differential effects in the majority of the data presented, it is not currently clear what the reduction of both nutrients down to physiological concentrations would do.
3. In Figure 1, the authors investigate expression of HIF target genes as well as of some cellular dioxygenases. It would be beneficial, given the later point the authors make (that HIF target gene expression is the product of a number of different activities), to investigate the expression of the dioxygenases that can directly alter HIF expression and activity; PHD1-3 and FIH.
4. The authors rightly point to the link between hypoxia-induced HIF-1α protein expression (Figure 3B) and transcriptional activity (Figure 3C) being complex. However, it is also clear that the 2-fold reduction in overall expression, and what appears to be a 50-fold reduction in reporter activity suggest a significant disconnect between the two. Given that the sub-set of well-described HIF1-target genes are differentially regulated – with most remaining unchanged (CA9, GLUT1, VEGFA and BNIP3) – the data suggest that the reporter is not providing physiologically-relevant information. It is not clear from the Materials and Methods section the nature of the reporter construct utilised – as it is almost certainly derived from a HIF-1α target gene promoter, was it one that was analysed in its endogenous setting (e.g. CA9, GLUT1, VEGFA, etc.)?
5. In the methylation analysis, the authors reported a lower mRNA expression of all three TET enzymes (Figure 3A), and note a decrease in hydroxymethylation of DNA (Figure 4). It should also

be noted that no increase in DNA methylation was observed (statistical test fell short of significance), which is therefore in contrast to the aberrant methylation previously reported in hypoxia (reference as cited in the manuscript). Could the authors incorporate a comment on this into the manuscript, or investigate why this is the case? To help support the authors' further comments on the effects of supraphysiological glucose concentrations on TET2 stability, the protein levels of the TETs could be presented under the conditions investigated. These data would help clarify this somewhat complex issue as it appears that in their system, oxygen concentrations *per se* do not alter global DNA methylation.

6. Finally the authors present data from HCC, where in contrast to the in vitro data presented, the TETs are not apparently co-ordinately regulated (Figure 5 and Figure 3A). It would be useful to clarify this in the manuscript, as the compensation suggested by the authors was not observed in their experimental system.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 08 October 2018

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This work by Lai *et al.* tells a very interesting story studying the effect of cellular nutrients availability, such as glucose and glutamine, on transcriptional responses to low oxygen. In general, the paper is well written and organized. I have following comments and suggestions:

1. Hypoxia inducible factors (HIFs) are a master regulator of oxygen sensing and define cellular transcriptional response to low oxygen level. Under hypoxia, the authors found the nutrients levels including glucose and glutamine, significantly affect the expression of alpha-ketoglutarate-dependent dioxygenases TETs (TET1, 2, 3), ALKBH5, and FTO. TETs are DNA demethylases which mediate DNA demethylation through the hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine, and 5-carboxycytosine, while ALKBH5 and FTO are mRNA N6-methyladenosine (m6A) demethylases. The authors only studied the hypoxia-driven change of TETs expression and the corresponding 5mC and 5hmC alterations in HepG2 cells. I suggest authors look at the mRNA m6A level change because mRNA m6A demethylases ALKBH5 and FTO are also significantly altered under hypoxia. The mRNA m6A modification has been known to post-transcriptionally affect mRNA abundance. The total mRNA expression level changes should arise from both transcriptional (DNA methylation involved) and post-transcriptional (mRNA methylation involved) layers. The investigation on RNA methylation may provide more insights.
2. In Figure 2A, the axis title ‘dCT’ should be presented as ‘DCt’, otherwise it is misleading.

The trend of TETs expression changes in Figure 3A and Figure 5 show inconsistency. Does it means that cell lines and tumor tissues have different environments?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 01 October 2018

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How the environmental impact, such as nutritional status and oxygen level, affect intrinsic transcriptome remain less clear. In this study, Lai et al took on this important challenge to demonstrate the nutrition supply and oxygen level in cell culture media play important roles in affecting global transcriptome and DNA methylome. The expression of several HIF target genes and Dioxygenases in HepG2 cells were altered in response to the change of Glucose and glutamine concentrations and oxygen levels in the media. Furthermore, the expression of HIF protein was altered in response to glucose and glutamine availability and positively correlated with its target expression. Importantly, altered dioxygenase expression, including TET proteins, resulted in global change of cytosine modification homeostasis. Finally, they found TET2 expression was also decreased in hepatocellular carcinoma, providing molecular mechanism underlying hypoxia-induced hypermethylation in liver cancer. Recent studies start to shed light on the link between HIF and TET proteins. For instance, HIF1A is significantly upregulated during mast cell activation, in which TET2 plays key roles in shaping epigenetic landscape. However, the roles of HIF-TET axis in response to nutritional status and cancer is not explored. Overall, this is a timely and well-tailored study addressing a fundamental yet under-developed question. The results could offer important molecular insights linking external environmental impact to intrinsic transcriptome and epigenome alteration, and their implications in carcinogenesis.

Specific comments:

1. The change of gene expression in Figure 2A was presented using dCT. Could the author use log2FC as presented in Figure 2B? These gene expression alterations were assessed under ambient oxygen conditions. Could the author test a few genes under normal DMEM condition?
2. In Figure 3, it is very interesting to demonstrate the change of HIF1a and test its effects using reporter gene bearing HRE, do they have a control reporter gene without HRE?
3. The authors found TET2 is specifically downregulated in liver cancer, which is in line with its established role as tumor suppressor. I am curious whether there are change of HIF1 target genes in these tumor samples?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.