Original Research

Characterization and comparison of hypoxia inducing factors on tumor growth and metastasis between two- and three-dimensional cancer models

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\textbf{A B S T R A C T}

The monocarboxylic acid transporter 4 (Mct-4), a downstream marker of hypoxia inducing factor (HIF)-1\textalpha, is involved in the cellular response to hypoxia, as indicated by the hypoxic response element in its promoter region. Using a tumorsphere assay as an in vitro three-dimensional (3D) model generated using 384-well ultra-low attachment (ULA) plates for cell proliferation analysis using a plate-based image cytometer, we identify a hypoxic response in the tumorsphere model that is distinct from that of cells grown under 2-dimensional (2D) normoxic conditions and demonstrate a key role for Mct-4 in enabling 3D growth. The tumorsphere model yields evidence of an essential role for Mct-4 in multiple cell lines, which were genetically modified to underexpress and overexpress Mct-4, evidence not apparent in a standard 2D model of growth in the same cell lines. In addition, we identify the effects of overexpressing Mct-4 in cancer cell migration using a transwell chamber assay. We also show that the response to hypoxia may be circumvented by transfection with a CMV promoter driven Mct-4, which confers constitutive 3D growth, wherein tumorsphere growth inhibition by small molecule HIF-1\textalpha inhibitors is mitigated. Finally, we demonstrate quantifiable gene/protein expression differences between 2D and 3D cancer models based on the normoxic and hypoxic conditions. Therefore, the tumorsphere 3D model generated using 384-well ULA plates in combination with high-throughput image cytometer is demonstrated to provide a convenient, robust, and reproducible tool and method for the elucidation of mechanisms of action underlying tumor growth and migration in the hypoxic tumor microenvironment.

1. Introduction

The Warburg effect involves the reprogramming of energy metabolism in cancer cells and has been recognized as a hallmark of cancer [1]. This effect, wherein cancer cells take up high amounts of glucose for glycolytic metabolism, ultimately yields excessive amounts of intracellular lactic acid via fermentative glycolysis. The production of intracellular lactic acid and concomitant lowering of intracellular pH are mitigated by cellular efflux of lactic acid via the monocarboxylic acid transporter (Mct) system [2]. Therefore, cellular glucose uptake via glucose transporters and lactic acid efflux via Mct comprise essential nodes in maintaining the unique metabolic processes driving cancer cell survival and proliferation.

Hypoxia is a prominent component of the tumor microenvironment in cancer cells and is a result of an imbalance between the increasing demand for oxygen and nutrients by a rapidly proliferating mass of tumor cells and the inefficient vascularization thereof, which characterizes tumor angiogenesis [3,4]. Hypoxia is a marker of poor prognosis in many types of human cancer, including breast, non-small cell lung, head and neck, ovarian and cervical cancer [5,6]. Furthermore, hypoxia mediates several biological processes critical for cancer progression, including angiogenesis, epithelial-mesenchymal transition (EMT), migration/invasion, and metastasis [7,8]. Therefore, hypoxia has been associated with reduced patient survival in many cancers [6].

A link between Mct-4 and hypoxia derives from the hypoxia response elements (HRE) in the promoter region of the Mct-4 gene [9,10], by which the translocation of hypoxia inducing factor (HIF)-1\textalpha to the nucleus drives expression of Mct-4 [11]. A role for Mct-4 in the cellular response to hypoxia can be inferred from the HRE, yet a functional delineation of this relationship remains unclear. Here we characterize the effects of HIF-1\textalpha and Mct-4 expression on the viability, growth, and mobility of cancer cells using in vitro two-dimensional (2D) and three-dimensional (3D) models for assessing tumor growth and metastasis [12]. Tumorsphere generation is a simplified 3D tumor model, where the cancer cells are cultured in surface treated round-bottom microplates, which can form into individual spheres due to the low attachment surface treatment in the wells. The standard two-dimensional (2D) cell culture was performed in flat-bottom microplates or flasks. Both cancer models can be assessed in a high-throughput manner using plate-based image cytometer, such as the Celigo Image Cytometer (Revvity Health Sciences, Inc., an indirect parent company of Nexcelom Bioscience, LLC., Lawrence, MA). Ishikawa, HCT116, HepG2,

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https://doi.org/10.1016/j.slasd.2023.10.007
Received 11 August 2023; Received in revised form 25 October 2023; Accepted 27 October 2023
Available online 30 October 2023
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and U87-MG cancer cells were used to generate 2D and 3D cell culture models to assess the effects of hypoxia inducing expressions on tumor growth, viability, and mobility. First, HIF-1α inhibitors were employed to inhibit hypoxia induced protein expression and to measure their effects on tumor viability and growth. Second, we characterized the effects of Mct-4 depletion and overexpression on tumor growth and invasion.

Next, we compared hypoxia inducing expressions between 2D and 3D models. Finally, we characterized mRNA transcriptions for different gene expressions between 2D and 3D models.

The results showed that 3D tumorsphere models, wherein cells are grown under normoxic cell culture conditions, reproduced essential elements of tumor growth with respect to the induction of both hypoxia and hypoxic response, which was not or minimally present in a normoxic 2D model. Using the tumorsphere model, Mct-4 is shown to mediate cell growth under hypoxic conditions induced by 3-dimensional cell growth. In addition, Mct-4 is shown to be essential to metastasis, as reproduced in a chamber model of TGF-β inhibitor-driven cell mobility. Therefore, HIF-1α, Mct-4, and other hypoxia induced mRNA and protein expression are herein demonstrated to be related and indeed critical to 3D tumor growth in the tumorsphere culture in multiple cell lines.

2. Materials and methods

2.1. Cell cultures

Ishikawa cell line was acquired from Millipore Sigma (Burlington, MA) and cultured in DMEM media (Gibco) supplemented with 10 % FBS and at 37 °C and 5 % CO₂. HCT116 cell line was acquired from ATCC (Manassas, VA) and cultured in McCoy’s 5A media (Gibco) supplemented with 10 % FBS at 37 °C and 5 % CO₂. The Panc1, U87-MG, and HepG2 cell lines were acquired from ATCC and cultured in DMEM media supplemented with 10 % FBS at 37 °C and 5 % CO₂.

2.2. Inhibition of hypoxia inducing expression using NTW-4975

SDS-PAGE was performed to measure the inhibitory effects of NTW-4975, an HIF-1α inhibitor, on HIF-1α, Mct-4, Hexokinase 2 (HK-2), and Pyruvate Dehydrogenase Kinase (PDK1), with GAPDH as the control. First, Ishikawa cells were seeded into 6-well plates and grown to ~80–90 % confluence and exposed to NTW-4975 at 5 μM for 24 h at 37 °C and 1 % O₂. After incubation, the cells were lysed and the lysates were subjected to SDS-PAGE. The blots were probed with antibodies to HIF-1α (GTX127309, GeneTex, Irvine, CA), Mct-4 (sc-376,465, Santa Cruz Biotecnology, Dallas, TX), HK-2 (#2867, Cell Signaling Technology, Danvers, MA), PDK1 (MA5-15797, Thermo Fisher Scientific, Waltham, MA), and GAPDH (#2118, Cell Signaling Technology). The gel image was captured using a Bio-Rad ChemiDoc Molecular Imager (Bio-Rad, Hercules, CA).

2.3. Effects of NTW-4975 on Ishikawa viability and growth in 2D and 3D models

NTW-4975 was previously identified from a screen of novel in-house proprietary compounds as inhibiting HIF-1α promoter driven luciferase in a cell-based hypoxia response assay. However, the mechanism of action is unknown.

To investigate the effects of NTW-4975 inhibitor on cell viability and growth, Ishikawa cells were seeded into 384-well flat-bottom and ultradeep-well plates at 2000 cells per well and incubated overnight at 37 °C and 1 % O₂. The flat- and round-bottom plates were used to generate 2D and 3D (tumorsphere) models, respectively. Subsequently, the cells were exposed to the HIF-1α inhibitor (NTW-4975) with serial dilution from 25.0 μM at 1:3 dilutions to 0.1 μM for 72 h at 37 °C and 5 % CO₂. Control wells were generated by treating the Ishikawa cells with BEZ235 (causes growth arrest) at 1 μM in 3D and Tamoxifen at 100 μM (highly cytotoxic), which were acquired from Sigma-Aldrich (St. Louis, MO).

After the 72-h incubation, the viability of Ishikawa cells was assessed using the CellTiterGlo ATP viability assay (Promega, Madison, WI) for both 2D and 3D models. Growth was also assessed using CellTiter Blue (CTB, Promega) and Celigo Image Cytometer for 2D and 3D models, respectively. The control wells were used to normalize the results and generate percent differences against the controls. In addition, the HIF-1α activity was measured with luciferase bioluminescence (OneGlo; Promega) in a HepG2 cell line transfected with a lentiviral HIF-1α promoter driven luciferase model (SA Biosciences, Frederick, MD).

2.4. Effects of Mct-4 depletion on HCT116 tumorsphere growth

Mct-4 depletion was accomplished with siRNA knockdown. First, HCT116 cells were seeded into two 6-well plates at 400,000 cells per well and allowed to grow to approximately 75 % confluence. The siRNA (10 μmol/well) was then added to 6 wells for Mct-4 depletion with 6 control wells. After 48 h of incubation, cells were confirmed with depleted Mct-4 expression by Western Blot (n = 6).

To investigate the effects of Mct-4 depletion (downstream protein pathway of HIF-1α) on cell growth in 2D and 3D models, HCT116 cells with or without siRNA Mct-4 depletion were seeded into 384-well flat-bottom and ULA round-bottom microplates at 2000 cells/well and incubated for 72 h at 37 °C and 5 % CO₂ (n = 6). To determine the Mct-4 expression in 2D, the cells were fixed and stained with Primary Mct-4 Ab (Abcam), Alexa Fluor 488 (Invitrogen) and GAPDH (Thermo Fisher Scientific) for 1 h, and analyzed on the Celigo Image Cytometer to measure the integrated fluorescent intensities. In addition, the CellTiterGlo ATP viability assay was performed in 2D. For the 3D model, the tumorspheres were imaged and analyzed on the Celigo Image Cytometer to measure tumor volume at 72 h.

2.5. Effects of Mct-4 depletion on TGF-β driven Panc1 invasion assay

Cell mobility in vitro can be measured with a chamber migration assay, wherein EMT-induced in vitro by TGF-β is assessed via measurement of cellular migration through a porous semi-permeable membrane. Therefore, it is important to evaluate the role of Mct-4 in an EMT-induced migration assay using Panc1 cells.

To investigate the effects of Mct-4 depletion on Panc1 invasion, Panc1 cells were grown to approximately 75 % confluence in 6 well plates and treated with control or Mct-4 siRNA as indicated. After 48 h of incubation, cells were confirmed with depleted Mct-4 expression by Western Blot (n = 6).

Next, approximately 2000 cells of control siRNA or Mct-4 siRNA transfected cells were then seeded into the top well of a 96-well Caltrex Cell Invasion Assay plate (R&D Systems, Minneapolis, MN). After 72 h incubation, the cells were stained with Calcein AM and the fluorescence of invading cells into the bottom chamber was determined per manufacturer’s instructions. Fluorescence was read on a Victor™ Nivo™ plate reader (PerkinElmer, Inc., Waltham, MA).

2.6. Generation of Ishikawa cell lines with overexpressing of Mct-4

Ishikawa cells were transfected to generate four clonal cell lines (#6, #8, #10, #11) that overexpress Mct-4. To confirm the transfection, SDS-PAGE was performed to measure Mct-4 protein content and GAPDH as the control. First, transfected clones were seeded into 6-well microplates and cultured for 24 h at 37 °C and 5 % CO₂ to approximately 75 % confluence. Subsequently, the cells were lysed and the lysates were subjected to SDS-PAGE. The blots were probed with antibodies to Mct-4, CD147 (Cell Signaling Technology), and GAPDH, and the image was captured and quantitated on a Bio-Rad ChemiDoc Molecular Imager.
2.7. Effects of Mct-4 overexpression on Ishikawa growth in 2D and 3D models

To investigate the effects of Mct-4 overexpression, Ishikawa cells of Clone #8 and #11 were seeded into 384-well flat-bottom and ULA round-bottom microplates at 2000 cells/well and incubated up to 96 h at 37 °C and 5 % CO₂. The tumorsphere volumes for Clone #8, #11, and non-transfected Ishikawa cells were monitored at 48, 72, and 96 h using the Celigo Image Cytometer. After the 72-h incubation, the viability of Ishikawa cells in 2D was assessed using the ATP viability assay.

2.8. Effects of Bay 87-2243 on Ishikawa growth with overexpressed Mct-4 in 2D and 3D models

Bay 87-2243 (#57309, Selleckchem, Houston, TX) is a HIF-1α inhibitor, which acts via inhibition of mitochondrial complex 1 [13]. To investigate the effects of Bay 87-2243 on tumor growth and viability, Ishikawa cells of Clone #11 were seeded into 384-well flat-bottom and ULA round-bottom microplates at 2000 cells/well and incubated for 18 h at 37 °C and 1 % O₂. Subsequently, the cells were exposed to Bay 87–2243 with serial titration from 1.000 μM at 1:3 dilutions to 0.004 μM for 72 h at 37 °C and 5 % CO₂. After the 72-h incubation, the ATP viability assay was performed for both 2D and 3D models.

2.9. Hypoxic protein expression comparison between 2D and 3D models

Protein expression levels of HIF-1α, Mct-4, and CD147 were measured to compare between 2D and 3D models using HCT116 and Ishikawa cells. First, HCT116 and Ishikawa cells were seeded into 384-well flat-bottom and ULA round-bottom microplates at 2000 cells/well and incubated for 5 days at 37 °C and 5 % CO₂. After 5 days, tumorspheres and 2D grown cells were respectively collected from the round-bottom plates using a Nanoscreen robotic liquid handler or from cell culture flasks, lysed, and the lysates were subjected to Western Blot. The blots were probed with antibodies to HIF-1α, Mct-4, CD147, and β-Actin. The gel image was captured and quantitated using a Bio-Rad ChemiDoc Molecular Imager.

2.10. Comparison of mRNA transcription between 2D hypoxia to 3D normoxia conditions

To compare differences of mRNA transcription in 2D and 3D models, Ishikawa cells were seeded into 384-well flat-bottom and ULA round-bottom microplates at 2000 cells/well, respectively. For the 2D model, the cells were allowed to proliferate in normoxia condition or hypoxia (1 % O₂) for 24 h. For the 3D model, the cells were allowed to incubate for 4 days at 37 °C and 5 % CO₂. The mRNA from 2D and 3D models were purified, where the cDNA was reverse transcribed and evaluated by the Hypoxia Signaling Pathway PCR Plus Array [14] (#330231, Qiagen, Germantown, MD) to measure the up- or down-regulation of SLC2A3, HIF3A, HPRT1, HNF4A, PCC, F10, and HK-2.

2.11. Comparison of glucose transporter mRNA transcription between 2D and 3D normoxia conditions

To compare differences of glucose transporter (Glut-1 and Glut-3) mRNA transcription in 2D and 3D models, Ishikawa cells, HCT116, or U87-MG were seeded into 384-well flat-bottom and ULA round-bottom microplates at 2000 cells/well, respectively. For the 2D model, the cells were allowed to proliferate in normoxia condition for 24 h. For the 3D model, the cells were allowed to incubate for 4 days at 37 °C and 5 % CO₂. The mRNA from 2D and 3D models were purified, where the cDNA was reverse transcribed and evaluated by the Hypoxia Signaling Pathway PCR Plus Array. HK-2 was also measured as comparison.

3. Results

3.1. Inhibition of hypoxia induced expression only affects Ishikawa growth and viability in 3D model

Ishikawa cells were exposed to NTW-4975 for 24 h under hypoxia condition to demonstrate its inhibitory effects on (1) hypoxia induced protein expression and (2) its downstream effects on cell viability and growth in 2D and 3D models. In addition, effects of NTW-4975 on HIF-1α promoter driven luciferase were characterized on the HepG2 cells. SDS-PAGE results showed noticeable dose dependent inhibitory effects of the hypoxia induced expression of HIF-1α, Mct-4, HK-2, and PDK1 in comparison to the GAPDH control (Fig. 1(a)). The results indicated that NTW-4975 is an effective inhibitor of HIF-1α and its downstream protein pathways.

Fig. 1(b) confirmed dose dependent inhibitory effects of NTW-4975 on HIF-1α promoter driven luciferase expression (IC₅₀ = 1.855 μM), as measured by luminescence in the HepG2 cell line. In the Ishikawa 2D model, the NTW-4975 showed minimum effects on cell growth measured by CellTiter Blue and no effects on cell viability measured by ATP assay (Fig. 1(b), (c)). On the other hand, the HIF-1α inhibitor showed clear dose response effects on both tumorsphere growth as measured by image cytometry and viability as measured by ATP assay in the Ishikawa 3D model with IC₅₀ values of 622.4 and 491.8 nM, respectively (Fig. 1(c)). In addition, tumorsphere growth assays were conducted in HCT116 and U87-MG cells, which formed compact tumorspheres suitable for quantitative imaging on the image cytometer. Similar results of NTW-4975 inhibiting tumorsphere growth were observed in HCT116 and U87-MG cells (data not shown).

3.2. Mct-4 depletion decreases HCT116 tumorsphere growth and Panc1 tumor invasion

Effects of Mct-4 depletion was characterized on HCT116 tumorsphere growth and Panc1 tumor invasion. Fig. 2(a) confirmed approximately 40 % reduction of Mct-4 fluorescence in fixed, permeabilized, and stained HCT116 cells. In the siRNA-treated HCT116 tumorspheres, the volume showed a reduction of approximately 30 % (Fig. 2(b)), whereas siRNA-treated HCT116 cells seeded into 2D plates showed no such difference between control and Mct-4 knockdown cells in an ATP viability assay (Fig. 2(c)).

SDS-PAGE results for the siRNA-mediated depletion of Mct-4 in Panc1 are shown in Fig. 2(d), where the Mct-4 showed close to complete depletion as compared to the control. Sister wells were transfected with control or Mct-4 siRNA as indicated and seeded into the top well of the chamber assay. Fig. 2(e) shows data from each of the control (n = 6) or Mct-4 (n = 6) siRNA transfected wells seeded into six chamber assay wells, with cells migrating into the bottom chamber at 48 h detected by Calcein AM. The results showed that TGF-β driven migration into the bottom chamber was inhibited in the Mct-4 siRNA treated cells, thus providing support for Mct-4 expression playing a role in EMT and cancer cell migration.

3.3. Mct-4 overexpression increases Ishikawa tumorsphere growth and reverses HIF-1α inhibitor effects

Effects of Mct-4 overexpression was characterized on Ishikawa cells in 2D and 3D models. Fig. 3(a) confirmed the overexpression of Mct-4 in stable Clone #8 and #11, which were derivatives of Ishikawa cells. Interestingly, these stable cell lines demonstrated overexpression of CD147 by SDS-PAGE, which corresponded with its known association to Mct-4 at the cell surface [15].

The time-dependent tumorsphere growth results in Ishikawa cells are shown in Fig. 3(b), demonstrating a significant increase of tumorsphere volumes in comparison to the control over the 96-h time frame. Both Clone #8 and #11 showed similar growth percentages at approximately
2.2X increase over the control. On the other hand, the endpoint 72-h ATP viability results showed minimal differences between Ishikawa Clone #8, #11, and the control in the 2D model (Fig. 3(c)).

The dose response results of Bay 87-2243 are shown in Fig. 3(d), (e). The results showed that Ishikawa Clone #11 with Mct-4 overexpression reversed or suppressed the inhibitory effects of Bay 87-2243 in the 3D model, which resulted in IC$_{50}$ values of 1.6 and 9.1 nM for the control and Clone #11, respectively. On the other hand, no visible differences were observed between the control and Clone #11 in the 2D model, which generated IC$_{50}$ values of 221.7 and 61.4 nM for the control and Clone #11, respectively.
3.4. Hypoxia inducing expression increases significantly in HCT116 and Ishikawa 3D models

Western Blot results shown in Fig. 4(a) demonstrate increases in protein expression for HIF-1α, Mct-4, and CD147. The β-Actin corrected protein levels are shown in Fig. 4(b), (c) for HCT116 and Ishikawa cells, respectively. The HCT116 cell type results showed a fold of increase (3D/2D) of 2.5, 4.6, and 9.0 for HIF-1α, Mct-4, and CD147, respectively. The Ishikawa cell type results showed a fold of increase of 4.0, 1.9, and 1.6 for HIF-1α, Mct-4, and CD147, respectively. Taken all together, hypoxia induced expression of key proteins is demonstrated and confirmed to increase in the 3D over 2D models.

3.5. Increase of mRNA transcription in 3D model under normoxic conditions

The mRNA transcription was measured using standard RT-PCR assay using Ishikawa cells in 2D model under normoxic or hypoxic conditions, where the fold changes were compared to Ishikawa cells under normoxic conditions. The fold changes were also calculated comparing between 3D model under normoxia to 2D model under hypoxia conditions. The results are shown in Table 1, which showed that mRNA transcription increased from approximately 1.8 to 4.4 folds from 2D to 3D model. In addition, the Glut-1 and Glut-3 for glucose transporter mRNA transcription, as well as the HK-2 showed increased fold changes from 2D to 3D models under normoxic conditions for Ishikawa, HCT116, and U87-MG (Fig. 5).

4. Discussion

Hypoxia plays a critical role in the growth and survival of solid tumors, wherein unfavorable conditions of oxygenation and vascularization in the tumor microenvironment require tumor cells to adapt in order to survive and proliferate. Major players in the hypoxic pathway include HIF-1α and Mct-4 as key hypoxia induced proteins that can promote tumor growth. In this work, we pharmacologically and genetically modulated HIF-1α and Mct-4 activity and expression to characterize their effects on tumor growth, viability, and mobility. In addition, multiple cell lines were selected to investigate the effects of hypoxia inducing factors based on their response to the Mct-4 depletion, overexpression, and migration.

When HIF-1α was inhibited by NTW-4975, the downstream hypoxia response mediators such as Mct-4, HK-2, and PDK1 were reduced. Mct-4, HK-2 [16], and PDK1 [17] are known to have a hypoxia response element in their respective promoter regions and to upregulate in response to hypoxia. A clear difference was observed between the 2D and 3D tumor models. The NTW-4975 showed dose dependent inhibition of growth and viability in 3D, while 2D did not exhibit noticeable changes, indicating an essential role of hypoxic response mediators in 3D culture.

Mct-4 is shown here to demonstrate the adaptive response of cancer cells to hypoxia as a key downstream mediator. The depletion of Mct-4 showed a significant decrease in tumor growth in 3D cell culture, while cell growth in standard 2D conditions was unaffected. Furthermore, we have observed that the depletion of Mct-4 eliminated the ability of tumor cells to mobilize in the invasion assay, showing less migration through a porous membrane towards TGF-β in the chamber migration assay.

In contrast, the overexpression of Mct-4 resulted in significantly enhanced tumor growth in 3D culture, while 2D did not show any noticeable effects. Furthermore, Mct-4 overexpression can reverse the tumorsphere growth inhibitory effects of HIF-1α inhibitor (Bay 87-2243), suggesting that the Mct-4 component of the HIF-1α adaptive response is essential to tumor growth under hypoxia, as its constitutive expression solely mitigates the effect of HIF-1α pharmacological shutdown on tumorsphere growth.
One of the most important characteristics of solid tumor is the tumor microenvironment (TME). The 2D and 3D cell culture format was employed to demonstrate the differences and importance of establishing a proper cell model that can better recapitulate the tumor microenvironment. We assumed that 3D tumorsphere model under normoxic conditions can yield a hypoxic response analogous to hypoxia within an actual tumor, which can be compared to cells grown in hypoxic chamber in 2D cell culture. The 3D cell culture demonstrated a significant increase in hypoxia induced mRNA and protein expression (HIF-1α, Mct-4, and CD147) versus the 2D format, which again highlights the differences between the two cell culture formats, where the 3D culture comprised of a hypoxia induced hypoxic response, thus enabling growth and survival in cultured tumorspheres. The mRNA transcription results showed increased gene expression compared between 3D and 2D cell culture, which further amplified the need to generate a proper cell culture model that can better approximate the tumor microenvironment. The mRNA signatures did vary between cell lines, with some commonly upregulated genes and some differentially upregulated. Interestingly, the gene signatures showed some variation between 3D cell culture grown under normoxia, and 2D cell culture grown under hypoxic conditions, suggesting unique properties in each in vitro model which should be

### Table 1

The mRNA expression fold changes of different genes/proteins showing differences between 2D in normoxia, 2D in hypoxia, and 3D in normoxia.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>2D Normoxia</th>
<th>2D Hypoxia</th>
<th>3D Normoxia</th>
</tr>
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<tr>
<td>SLC2A3</td>
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<td>4.39</td>
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<td>2.62</td>
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<tr>
<td>HK2</td>
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Fig. 4. (a) Western Blot showing increase in hypoxia induced protein expression grown in 2D and 3D conditions \((n = 2)\), which are quantified with the β-Actin normalized protein levels for (b) HCT116 and (c) Ishikawa cells. Quantitation was performed using ImageLab™ (BioRad), where data is shown from one experiment.

Fig. 5. The mRNA expression fold changes of Glut-1, Glut-3, and HK-2 for Ishikawa, HCT116, and U87-MG cell lines between 3D and 2D models. Experiment was performed in duplicate, but data is shown from one experiment.
considered when attempting to correlate mRNA and protein expression to phenotypic parameters. It is important to note that there is inherent variability in the RT-PCR assays that may cause some variations in the levels of fold changes.

In conclusion, we have characterized and compared the effects of hypoxia response factors, specifically HIF-1α and Mct-4, on tumor growth, viability, and mobility, between 2D and 3D cell culture models. Demonstration of differences between 2D and 3D models suggests the importance of the 3D model in recapitulating the tumor microenvironment. With the employment of high-throughput image cytometers, both 2D and 3D models may be easily evaluated rapidly and reliably. Ultimately, in order to generate an appropriate tumor model for identifying candidates for small molecules, antibodies, or cellular therapies on solid tumors, it may be useful to employ an in vitro 3D cell culture model that can recapitulate hypoxic response activities that are more representative and physiologically relevant.

Declaration of Competing Interest

The authors LLC, SLK, and BL declare competing financial interests. The Celigo Image Cytometer used in this work to assess tumorsphere growth, invasion, and fluorescence intensities is a product of Revvity Health Sciences, Inc. (an indirect parent company of Nexcelom Biosciences, LLC.), Lawrence, MA.

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