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HYPOXIA AND SUCCINATE ANTAGONIZE 2-DEOXYGLUCOSE EFFECTS ON GLIOBLASTOMA

Francesca Pistollato^{1†}, Sara Abbadi^{1†}, Elena Rampazzo¹, Giampietro Viola^{1*}, Alessandro Della Puppa², Lucia Cavallini³, Chiara Frasson¹, Luca Persano¹, David M. Panchision⁴ and Giuseppe Basso¹.

¹*Hemato-Oncology Laboratory, Department of Pediatrics, University of Padova, Italy*

²*Department of Neurosurgery, University of Padova, Italy*

³*Department of Biochemistry, University of Padova, Italy*

⁴*Division of Neuroscience and Basic Behavioral Science, National Institute of Mental Health, National Institutes of Health, Bethesda, MD*

[†] Equal contributing authors.

*Address correspondence to: Giampietro Viola, Hemato-Oncology Laboratory, Department of Pediatrics, University of Padova, Via Giustiniani 3, Padova 35128, Italy. Tel: +39 049 8211451; Fax: +39 049 8211462; E-mail: giampietro.viola.1@unipd.it

Abstract

Glioblastoma multiforme (GBM) are highly proliferative brain tumors characterized by a hypoxic microenvironment which controls GBM stem cell maintenance. Tumor hypoxia promotes also elevated glycolytic rate, thus limiting glucose metabolism is a potential approach to inhibit tumor growth. Here we investigate the effects mediated by 2-deoxyglucose (2-DG), a glucose analogue, on primary GBM-derived cells maintained under hypoxia. Our results indicate that hypoxia protects GBM cells from the apoptotic effect elicited by 2-DG, which raises succinate dehydrogenase activity thus promoting succinate level decrease. As a consequence hypoxia inducible factor-1 α (HIF-1 α) degradation occurs and this induces GBM cells to acquire a neuronal committed phenotype. By adding succinate these effects are reverted, as succinate stabilizes HIF-1 α and increases GBM stem cell fraction particularly under hypoxia, thus preserving the tumor stem cell niche. 2-DG inhibits anaerobic glycolysis altering GBM cell phenotype by forcing tumor cells into mitochondrial metabolism and by inducing differentiation.

Keywords: 2-deoxyglucose; Hypoxia; Hypoxia Inducible Factor-1 α ; proline hydroxylase 2; GBM differentiation.

Abbreviations.

GBM, glioblastoma multiforme; BMP2, bone morphogenetic protein2; HIF-1 α ., hypoxia inducible factor 1 α ; PHD2, Proline hydroxylase2; SDH, succinate dehydrogenase

1.Introduction

1
2 Brain tumors and particularly glioblastoma multiforme (GBM) are highly proliferative
3
4 tumors characterized by a hypoxic microenvironment. Recent literature shows that this
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6 hypoxic microenvironment correlates with tumor aggressiveness [1-3] and over-activity of
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8 hypoxia inducible factor-1 α (HIF-1 α) [4] is implicated in tumor progression and in the
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10 maintenance of a de-differentiated (i.e. stem) tumor cell phenotype [3]. Particularly, HIF-1 α
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12 has been found to contribute to a differentiation defect in malignant gliomas [5], even
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14 following exogenous bone morphogenetic protein2 (BMP2) treatment, known to induce glial
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16 commitment in GBM [6-8]. These evidences shed new light on the differentiation therapy of
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18 solid tumors by HIF-1 α targeting. Hypoxia has a role in normal physiological responses, such
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20 as carotid body growth and generation of new neural crest derived glomus cells [9], and
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22 angiogenesis [10]. It is implicated in the regulation of several developmentally important
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24 signaling pathways, such as Notch [11] and BMP pathways [7], as well as glucose
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26 metabolism [12]. Moreover, hypoxia alters cancer cells metabolism by increasing tumor
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28 anaerobic glycolysis and reducing mitochondrial activity [13]. Particularly SDH, the enzyme
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30 complex II bound to the inner mitochondrial membrane that converts succinate to fumarate
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32 via FAD reduction to FADH₂, has been found inactive in some solid tumors [14]. This has
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34 been described as a key event leading to the accumulation of intra-cytoplasmic succinate and
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36 reactive oxygen species (ROS) causative of the “pseudo-hypoxic” effect, a major tumor-
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38 related hallmark, characterized by the activation of HIF even under normoxia [14, 15]. As
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40 intra-tumoral hypoxia increases glycolytic activity in many types of cancer, including GBM,
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42 limiting glucose uptake or glucose metabolism is a promising therapeutic approach for cancer
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44 treatment[16, 17] in order to reduce tumor growth as shown in animal models [18]. 2-
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46 deoxyglucose (2-DG) is a glucose analogue which is converted by hexokinase to
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48 phosphorylated 2-DG, inhibiting glycolysis. Inhibition of glycolysis by phosphorylated 2-
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1 DG, 2-DG-6-phosphate, is thought to occur via inhibition of hexokinase and
2 phosphohexoisomerase [19,20] 2-DG is effective in several mouse tumor models, particularly
3 in combination therapy [17]. Indeed, metformin, a widely used antidiabetic agent, has been
4 seen to exert antitumoral and antiproliferative action in prostate cancer cells, especially when
5 used in combination with 2-DG [21]. Moreover, earlier studies have shown that 2-DG
6 significantly enhances the cytotoxic effects of anticancer agents like topoisomerase inhibitors
7 and a radiomimetic drug (bleomycin) in established human tumor cell lines. Also, it has been
8 shown that 2-DG can be used as a modulator of radiation response [22,23]. These studies
9 demonstrate that targeting carbohydrate metabolism may be an effective therapeutic
10 approach. Here we investigated how 2-DG affects primary GBM-derived cells growth, by
11 promoting differentiation and an increase in SDH activity, leading to HIF-1 α degradation.
12 Addition of succinate and preserving cells in a hypoxic microenvironment limit 2-DG effects
13 by maintaining GBM in an undifferentiated status. Finally, our results unravel a novel
14 mechanistic control on GBM cell metabolism and phenotype by 2-DG, succinate and oxygen
15 tension.
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39 **2. Materials and Methods**

40 *2.1 Isolation and gas-controlled expansion of cells*

41 Under institutional-approved protocols, tissue samples were collected from patients and
42 neonates after written informed consent. Neonatal brain subventricular zone (SVZ) tissue was
43 obtained at the Children's Hospital of Orange County, USA, under the auspices of the
44 protocol for the National Human Neural Stem Cell Resource (NHNSCR). Adult GBM
45 samples were obtained at the Padova Academic Hospital, under the auspices of the protocol
46 for the acquisition of human brain tissues obtained from the Ethical Committee board of the
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1 University of Padova. Tumor and non-tumor patients characteristics are described in Table
2 S1 (see Supplementary Information).
3

4 We dissociated and cultured cells as previously described [7]. Briefly, cells were cultured in
5 control medium consisted of HAM'S-F12/DME with 25 mM glucose, (Irvine Scientific,
6 Santa Ana, CA), BIT9500, 10%, as serum substitute (Stem Cell Technologies, Milano, Italy).
7 For continuous expansion, one-half of the medium was replaced every day and cells were
8 passaged every 7-10 days using TrypLE (Invitrogen, Carlsbad, CA) to dissociate them. Cells
9 were not cultured for more than 8 passages *in vitro* in order to avoid long term culture related
10 effects. Cell viability was evaluated by trypan blue exclusion. For low glucose experiments,
11 cells were cultured in DMEM/F12, glucose-free, (Gibco BRL, Milano, Italy) with BIT9500
12 (10%), for a final glucose concentration of 2.5 mM. Two different glucose concentrations
13 were established by adding D-glucose (12.5 and 25 mM final concentration). In experimental
14 groups 2-deoxyglucose (2-DG) (Sigma, Milano, Italy) was added to a final concentration of
15 12.5 or 25 mM as reported in literature [24, 25]. Cells were maintained in an atmosphere of
16 5% carbon dioxide and 2% (Ruskinn C300, Ruskinn Technology Ltd, Bridgend, UK) or 20%
17 oxygen balanced with nitrogen [7]. In some experiments, esterificated diethyl-succinate (5
18 mM, Sigma-Aldrich, Milano, Italy) or pyruvate (1 mM, Sigma-Aldrich, Milano, Italy) were
19 added for 18 hr. Pyruvate, the physiological substrate of pyruvate dehydrogenase (PDH), was
20 used to confirm specificity of antibody specific for PDH E1 α .
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49 2.2 Immunocytochemistry

50 Cells were fixed in cold 4% paraformaldehyde for 15 min, rinsed and stored prior to analysis.
51 Primary antibody staining was performed for nestin (mouse, 1:200, Millipore, Billerica, MA),
52 glial fibrillary acidic protein (GFAP, mouse, 1:1000, Sigma-Aldrich, Milano, Italy), β -III-
53 tubulin (mouse, monoclonal Tuj-1, 1:1000, Covance, Milano, Italy). After incubation, cells
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1 were washed and incubated with species-specific secondary antibodies conjugated to Alexa
2 dyes (1:2000, Invitrogen, Milano, Italy). Cells were counterstained with DAPI (1:10000,
3 Sigma-Aldrich, Milano, Italy) to measure total cell number. Staining was visualized by
4 epifluorescence (Nikon Eclipse 80i, Melville, NY, USA) and images compiled for figures
5 using Adobe Illustrator (Adobe, San Jose, CA).
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11 12 13 14 *2.3 CD133, CD90, cell cycle and Annexin-V/Propidium Iodide analyses*

15 Cells (2×10^5 cells/ml) were incubated with anti-human CD133 (clone AC133/2, PE
16 conjugated, (Miltenyi Biotec, Bologna, Italy) as described in [26] and anti-human CD90
17 clone 5E10, FITC conjugated, (BioLegend, Milano, Italy). Viability was assessed by adding
18 7-amino-actinomycin-D (7-AAD, 50 ng/ml, BD Biosciences, Milano, Italy) prior to analysis.
19 Cells were analyzed on a Cytomics FC 500 flow cytometer (Beckman Coulter, Milano, Italy).
20 Relative percentages of different subpopulations were calculated based on live gated cells.
21 Unlabeled cells and cells incubated with appropriate isotypic control antibodies were first
22 acquired to ensure labeling specificity. For cell cycle phase analysis, cells were incubated
23 with BrdU (1 mM, BD, Milano, Italy) for 45 min then washed and prepared for analysis as
24 directed (BrdU Flow Kit Staining Protocol, BD, Milano, Italy). Analysis of apoptosis was
25 performed as recommended by using the Annexin Fluo kit (Roche Biochemicals, Milano,
26 Italy).
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46 *2.4 Mitochondrial membrane potential, reactive oxygen species (ROS) and GSH analyses*

47 The mitochondrial membrane potential was measured with the lipophilic cation 5,5',6,6'-
48 tetrachlo-1,1',3,3'-tetraethylbenzimidazol-carbocyanine (JC-1, Invitrogen, Milano, Italy), as
49 described in [27], a fluorescent probe known to selectively enter into the mitochondria and
50 used to measure changes in mitochondrial membrane potential ($\Delta\Psi_{\text{mt}}$). JC-1 forms aggregates
51 (red color) at higher potential and monomers (green color) at low membrane potential. To
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1 ensure JC-1 effectiveness GBM-derived cells have been pretreated with valinomycin (1 μ M,
2 Sigma-Aldrich, Milano, Italy), facilitating mitochondrial membrane potential depolarization
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4 (not shown). The production of ROS was measured by flow cytometry using hydroethidine
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6 (HE) (Invitrogen, Milano, Italy) [27]. Analysis of total GSH level was measured by using
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8 monobromobimane (mBrB), (Invitrogen, Milano, Italy) (50 μ M) [28, 29] and chloromethyl-
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10 dichlorofluorescein diacetate (CM-DCFDA) (5 μ M, Invitrogen, Milano Italy), preincubating
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12 cells for 15 and 30 min at 37 °C respectively prior to analysis.
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19 *2.5 Western blot analyses*

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21 Total protein extracts were isolated in lysis buffer prepared as follows: TPER Reagent
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23 (Pierce, Milano, Italy), NaCl 300 mM, orthovanadate 1 mM (Sigma, Milano, Italy), AEBSF 2
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25 mM (PEFABLOC, Roche Biochemicals, Milano, Italy), Aprotinin 1 μ g/mL (Sigma-Aldrich,
26
27 Milano, Italy), Pepstatin-A 5 μ g/ml (Sigma-Aldrich, Milano, Italy) and Leupeptin 1 μ g/ml
28
29 (Sigma-Aldrich, Milano, Italy). Equal amounts of protein (10 μ g) were resolved using SDS-
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31 PAGE gels and transferred to PVDF Hybond-p membrane (GE Healthcare, Catania, Italy).
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33 Membranes were blocked with I-blockTM Blocking (Tropix, Applied Biosystem, Monza,
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35 Italy) for at least 2 hr under rotation at room temperature. Membranes were then incubated
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37 with primary antibodies against HIF-1 α (mouse, 1:250, BD Biosciences, Milano, Italy), HIF-
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39 2 α (mouse, 1:250, Novus Biologicals, Milano, Italy), PHD2 (goat, 1:300, Santa Cruz, Santa
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41 Cruz, CA, USA), p21^{cip1} (mouse, 1:1000, Sigma-Aldrich, Milano, Italy), Bcl-2, Bcl-XL,
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43 PARP, (all rabbit, 1:1000, Cell Signaling, Milano, Italy), phosphorylated PDH E1 α serine
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45 293 (rabbit, 1:5000, Novus Biologicals, Milano, Italy), total PDH E1 α subunit (1:1000,
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47 Invitrogen, Carlsbad, CA, USA) or β -actin (mouse, 1:10000, Sigma-Aldrich, Milano, Italy)
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49 for 2 hr. Membranes were next incubated with peroxidase-labeled goat anti-rabbit IgG
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51 (1:100000, Sigma-Aldrich, Milano, Italy), peroxidase-labeled goat anti-mouse IgG
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1 (1:100000, Sigma-Aldrich, Milano, Italy) or peroxidase-labeled donkey anti-goat IgG
2 (1:100000, Santa Cruz, Santa Cruz, CA, USA) for 60 min. All membranes were visualized
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4 using ECL Advance (GE Healthcare, Catania, Italy) and exposed to Hyperfilm MP (GE
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6 Healthcare, Catania, Italy).
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10 11 12 *2.6 Lactate Assay*

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14 Extracellular lactate measurement was performed using the Lactate Assay Kit, (BioVision,
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16 Torino, Italy) kindly provided by Dr. Alberto Burlina (University of Padova), following the
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18 manufacturer instructions.
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24 25 *2.7 Succinate dehydrogenase activity*

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27 To evaluate aerobic metabolism succinate dehydrogenase (SDH) activity has been measured,
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29 since enzyme activity is considered an indicator of tricarboxylic acid (TCA) cycle activity.
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31 To measure SDH activity, we used the methods described by Levine et al. with little
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33 modifications [30]. Briefly, cell monolayers (not fixed) were snap-frozen at -80°C for at least
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35 1 hr and were incubated for 20 min in the dark at 37°C in a reaction buffer containing 12.3
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37 mM diethyl-succinate (Sigma, Milano, Italy), 0.2 mM 1-methoxy-5-methylphenazine methyl
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39 sulphate (Sigma-Aldrich, Milano, Italy), 1.2 mM nitro blue tetrazolium (NBT, Sigma-
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41 Aldrich, Milano, Italy) in 50 mM Tris-HCl, pH 7.6. We used 12.3 mM malonate (Sigma,
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43 Milano, Italy) to test aspecific reactions, and 3-nitropropionic acid (3-NPA, 500 µM, Sigma-
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45 Aldrich, Milano, Italy), to irreversibly inactivate SDH, as negative control. The enzyme
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47 activity is determined by measuring the formation of nitro blue diformazan (NBT-dfz) due to
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49 NBT reduction and the end product (i.e. blue cells) is visible by bright field microscopy or
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51 measured at 570 nm, by absorption spectroscopy.
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2.8 ATP assay

Cells were cultured with 2-DG for 18 or 24 hr at either 2% or 20% oxygen, collected and counted. The ATP content per 100000 cells was determined using the CellTiter-Glo luminescent assay (Promega, Milano, Italy) according to manufacturer instruction, using a Victor³™ luminometer (Perkin Elmer, Milano, Italy). Data were normalized to ATP content in cells cultured with glucose at 2% O₂.

2.9 MTT assay

Individual wells of a 96-well tissue culture microtiter plate (Falcon, BD, Milano, Italy) were inoculated with 100 µl of medium containing 5x10³ of GBM cells. The plates were incubated overnight prior to the analyses. After medium removal, 2-DG was added and cells were incubated for 18 or 24 hr at either 2% or 20% oxygen. Cell viability was assayed by the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] test as previously described [31].

2.10 Real-Time PCR analysis

RNA was isolated from cells using Trizol (Invitrogen, Milano, Italy) and 1 µg of total RNA reverse-transcribed using SuperScript RNase H-Reverse Transcriptase (Invitrogen, Milano, Italy). Quantitative RT-PCR reactions were run in triplicate using Brilliant® SYBR® Green QPCR Core Reagent Kit (Stratagene, La Jolla, CA, USA). Fluorescent emission was recorded in real-time (Sequence Detection System 7900HT, Applied Biosystems, Foster City, CA, USA). Gene expression profiling was completed using the comparative Ct method of relative quantification. PCR amplification conditions consisted of 50 cycles with primers annealing at 60°C. These set of primers were used: *PHD2*, F: 5'-GGGACATTCATTGCCTCACT-3', R: 5'-ACACATGTGGTGCTTGCTGT-3'; *HIF-1α*, F: 5'-CGTTCCTTCGATCAGTTGTC-3',

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R: 5'-TCAGTGGTGGCAGTGGTAGT-3'; *beta-glucuronidase* (*GUSB*), F: 5'-GAAAATACGTGGTTGGAGAGCTCATT-3', R: 5'-CCGAGTGAAGATCCCCTTTTTA-3'. Primers have been designed by using the software Primer 3 (<http://frodo.wi.mit.edu/primer3/input.htm>) and the specificity of the primers for *GUSB*, *HIF-1 α* and *PHD2* sequences has been evaluated by using the software Human BLAT Search (<http://genome.ucsc.edu/cgi-bin/hgBlat?command=start>). PCR amplicons had been previously evaluated on agarose gel, and during SYBR green analyses primers dissociation curves have been checked in each run to ensure primers specificity. Relative RNA quantities were normalized to *GUSB* as a housekeeping gene and 2% oxygen T0 was used as the calibrating condition ($\Delta\Delta\text{Ct}$ Method).

2.11 Hypoxia Responsive Element (HRE)-luciferase reporter assay

GBM cells were transfected using a Promega Corporation (Madison WI, USA) protocol for transient transfection of adherent cells using Effectene reagent (Qiagen, Milano, Italy). HRE-luciferase reporter construct used (wHRE) was kindly provided by Dr. Stefano Indraccolo (Istituto Oncologico Veneto – IRCCS, Padova, Italy). It consists of a trimerized 24-mer containing 18 bp of sequence from the PGK promoter including the HRE (5'-TGTCACGTCCTGCACGACTCTAGT, HRE) and an 8-bp linker sequence followed by a 50-bp minimal tyrosine kinase promoter in a pGL2-firefly luciferase basic Vector backbone (Promega, Madison, WI, USA) [32]. The mutant HRE (mHRE) construct used to evaluate aspecific effects, has the ACG of the HIF-1 binding site mutated to CAT, abolishing binding, as well as a point mutation that abolished a BsgI restriction site for diagnostic purposes. GBM cells were transfected either with a wHRE or with a mHRE. Along with these vectors, also a Renilla luciferase vector has been transfected in order to normalize luciferase detection (Promega, Madison, WI, USA). 12 hr after transfection, total medium change was done and

1 cells were treated with 25 mM 2-DG, succinate 5 mM or a combination of both for 8 hr.
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3 Finally, cells were processed and analysis of HRE-luciferase activity was performed as
4
5 described (Dual-Luciferase Reporter Assay System, Promega, Madison, WI, USA) by using a
6
7 plate-reading luminometer (Victor³™, Perkin Elmer, Milano, Italy).
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10 11 12 *2.12 Statistical analysis*

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14 Graphs and statistical analyses were prepared using Prism 3.03 (Graph Pad, La Jolla, CA).
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16 All values were presented as mean \pm standard error of the mean (S.E.M.). Statistical
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18 significance was measured by one-way ANOVA with post-hoc Newman-Keul's test, *p <
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20 0.05, **p < 0.01, ***p < 0.001. For all graphs, an asterisk over a bracket indicates a
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22 significant difference with a variable as indicated, an asterisk over a bar indicates a
23
24 significant difference between the 2% and 20% oxygen at either a particular time point or
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26 culture condition.
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31 32 33 **3.Results**

34 35 *3.1 2-DG induces loss of GBM cell adhesive properties and decreases cell proliferation.*

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37 It has been suggested that hypoxia is one of the major regulatory factors in the brain tumor
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39 niche, since it positively correlates with brain tumor aggressiveness, supporting the idea that
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41 hypoxia may confer a growth advantage to brain tumor stem cells [2, 3, 5, 7]. Under hypoxia
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43 cancer cell metabolism is pushed toward increased anaerobic glycolysis, which has been
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45 extensively described as a hallmark of tumor cell biology [13]. Here, we used primary
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47 cultures of adult GBM tumors to study the interaction between oxygen levels and the effects
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49 elicited by 2-DG, thereby inducing a block of glycolysis. We evaluated cells grown in 2%
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51 oxygen, which represents the condition typically found in hypoxic solid tumors [33], versus
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53 cells that were acutely exposed to higher oxygen tension (20% oxygen). We found that
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addition of 25 mM 2-DG for 18 hr, under the two oxygen tensions, induced loss of cell
adhesive properties compared to GBM cells grown without 2-DG (Fig. 1A and Suppl. Fig.
1A,B). Simply decreasing the glucose medium concentration from 25 mM to 2.5 mM,
without adding 2-DG (Suppl. Fig. 1C) did not cause change of cell morphology. By adding
both 2-DG and glucose in the medium (i.e. 25 mM 2-DG + 25 mM glucose, Suppl. Fig. 1C,
or 12.5 mM 2-DG + 12.5 mM glucose, not shown), we found that loss of cell adherence was
prevented and cells showed a flat phenotype with more extensions compared to cells grown
in the absence of 2-DG. Measurement of extracellular lactate was generally high in tumor cell
culture medium (Fig. 1B) compared to non-tumor subventricular zone (SVZ)-derived cells
(Fig. 1C), consistent with GBM cell metabolic dependence on anaerobic glycolysis. Addition
of 2-DG under both oxygen tensions led to a strong extracellular lactate decrease in both
GBM and SVZ-derived cells (Fig. 1B, C). Moreover, 2-DG lowered GBM cells growth (Fig.
1D), especially in cells acutely exposed to high oxygen, while SVZ cells did not undergo
morphological changes (Suppl. Fig. 2A) and underwent proliferation reduction only when
acutely exposed to high oxygen tension (Fig. 1E).

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Analysis of GBM cell viability by trypan blue revealed no significant differences among
conditions (not shown), while methyltetrazolium reduction (MTT) (Fig. 1F), indicative of
extra-mitochondrial NADH and NADPH [34] occurred. Importantly, re-addition of glucose
after 2-DG exposure did not recover GBM cells growth (data not shown). Also, intracellular
ATP content in GBM cells, which was found higher at 20% oxygen tension consequentially
to stimulated mitochondrial activity, was decreased following addition of 2-DG (Fig. 1G).

54 55 56 57 58 59 60 61 62 63 64 65 *3.2 2-DG increases succinate dehydrogenase and pyruvate dehydrogenase activity.*

We sought to investigate if 2-DG, besides determining a glycolytic block, was able to perturb
mitochondrial metabolism. Thus, we analyzed succinate dehydrogenase (SDH) activity. It is

1 known that SDH activation is impaired in several solid tumors, inducing the accumulation of
2 succinate and ROS into the cytoplasm and causing strong HIF-1 α stabilization even in the
3 absence of hypoxia [14]. Surprisingly, we found that addition of 2-DG increased SDH
4 activity in GBM-derived cells, especially at 20% oxygen (Fig. 2A, B). Addition of cell
5 permeable succinate either in presence or absence of 2-DG did not modify SDH activation
6 state (data not shown). Notably, in SVZ-derived cells SDH activity did not change after
7 addition of 2-DG (data not shown), confirming differences in metabolic response between
8 non-tumor and tumor cells.
9

10 Additionally, we analyzed the phosphorylation state of pyruvate dehydrogenase E1 α (pPDH
11 E1 α) in order to test if addition of 2-DG induced an increase of mitochondrial metabolism.
12 The PDH complex contributes to transforming pyruvate into acetyl-CoA, which is used in the
13 citric acid cycle to carry out cellular respiration. The activation state of PDH is controlled by
14 the enzyme pyruvate dehydrogenase kinase (PDK), which inhibits PDH activity by
15 phosphorylating it using ATP [35]. We found that GBM cells maintained at 2% oxygen had a
16 higher level of pPDH compared to cells acutely exposed to 20% oxygen (see T0 at 2% and
17 T0 at 20% oxygen in Fig. 2C), indicating inactivation of the complex and increased lactate
18 production level under hypoxia, as confirmed in Fig. 1B. After the addition of 2-DG, a
19 transient increase of pPDH level occurred, which decreased after 18 hr (Fig. 2C) and 24 hr
20 (data not shown). Importantly, total PDH level did not significantly change following
21 addition of 2-DG (Fig. 2C). These results, together with the observation of an increase in
22 SDH activation after the addition of 2-DG (Fig. 2A, B) indicate a transient recovery of
23 mitochondrial activity following addition of 2-DG.
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25 As intracellular succinate level and ROS production are associated to SDH activity and
26 defects of electron transfer in the respiratory chain are bound to enhanced ROS production
27 [36], we evaluated the possible effects of 2-DG on ROS production. We found that hypoxic
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1 GBM-derived cells, which resulted to contain a variable but generally high percentage of
2 ROS, underwent transient superoxide anion ($O_2^{\cdot-}$) reduction after the addition of 2-DG for 18
3 hr (Fig. 2D). Importantly, by adding succinate to GBM cells ROS levels were higher under
4 both oxygen tensions and this effect was repressed by the addition of 2-DG (Fig. 2E).
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6 Conversely, significant variations of ROS content under the same conditions were not
7 observed in SVZ-derived cells (Suppl. Fig. 2B).
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15 The decrease in ROS may also relate to higher reduced glutathione (GSH) level. GSH is
16 maintained in cells by reduced nicotinamide adenine dinucleotide phosphate (NADPH),
17 which is primarily generated when glucose is metabolized by pentose phosphate pathway. By
18 measuring total GSH level we could not find any significant change following addition of 2-
19 DG (Suppl. Fig. 3). This suggests that ROS level reduction is primarily connected to higher
20 SDH activity (i.e. lower succinate level) rather than improved cytoplasmic radical
21 scavenging.
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32 33 34 35 *3.3 2-DG promotes HIF-1 α degradation in GBM cells.*

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37 It has been previously reported in a fibrosarcoma cell line that the hypoxic accumulation of
38 HIF-1 α , an endogenous hypoxia marker, strongly depends on glucose availability, as shown
39 by modulation with 2-DG[37]. Nevertheless, the molecular mechanism causing this
40 phenomenon has not been elucidated. HIF-1 α is known to be chemically stabilized by
41 hypoxia independent factors, such as mitochondrial ROS [38], $CoCl_2$, Fe^{2+} and cytoplasmic
42 succinate [14], all known to be cofactors and inhibitors of proline hydroxylases (PHDs) [39],
43 which target HIF-1 α degradation through proteasome. Importantly, we found that HIF-1 α
44 protein level was reduced by 2-DG under hypoxia, in some cases below detection level (Fig.
45 3A). Higher-molecular-weight isoform of HIF-1 α , indicative of poly-ubiquitylated isoform
46 [15], eventually occurred (Fig. 3A). In SVZ-derived cells HIF-1 α was rapidly reduced
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1 following addition of 2-DG but its recovery was visible after 18 hr (Suppl. Fig. 2C). HIF-2 α
2 has been described as a promising target for anti-GBM therapies as it has been found highly
3 expressed in glioma stem cells [40]. We found that, oppositely to HIF-1 α , HIF-2 α level was
4 not significantly decreased by addition of 2-DG (Fig. 3A).
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9 To explain HIF-1 α degradation, we tested expression of proline hydroxylase-2 (PHD2), the
10 best described proline hydroxylase involved in HIF-1 α protein hydroxylation. We found that
11 after addition of 2-DG, PHD2 protein increased under hypoxia (Fig. 3A) reaching the level
12 observed in cells exposed to 20% oxygen. We hypothesized that 2-DG-mediated PHD2
13 protein increase was dependent on *de novo* protein synthesis. Analyses of QRT-PCR revealed
14 that *HIF-1 α* transcript did not change after addition of 2-DG (Fig. 3B), while expression of
15 *PHD2* transcript was decreased by 2-DG (Fig. 3C), leading to exclude *de novo* PHD2
16 synthesis. Importantly, by silencing HIF-1 α with lentiviral vectors GBM-derived cells
17 acquired a more differentiated phenotype, with increased endogenous BMP signalling, and
18 eventually underwent cell death after 5-7 days, as already reported [7, 8]. Importantly, further
19 addition of 2-DG did not promote any difference in HIF-1 α silenced cells (data not shown).
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39 *3.4 Addition of succinate prevents 2-DG-mediated HIF-1 α degradation.*

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41 As HIF-1 α stability is related to accumulation of intra-cytoplasmic succinate consequential to
42 impaired SDH activity [14], we hypothesized that 2-DG dependent HIF-1 α degradation may
43 be caused by the observed increase of SDH activity (Fig. 2A,B). To prove this hypothesis we
44 evaluated if addition of succinate in combination with 2-DG, promoted a recovery of HIF-1 α
45 protein level in GBM-derived cells. We found that HIF-1 α stability was recovered in 2-
46 DG/succinate cultured cells (Fig. 3D). Analysis of HIF-2 α did not reveal any significant
47 change among conditions (Fig. 3D).
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1 We investigated if 2-DG, besides modulating HIF-1 α protein stability, promoted also
2 inhibition of HIF-1 α transcriptional activity, by using a hypoxia responsive element (HRE)-
3 luciferase reporter construct. We found a modest reduction of HIF-1 α transcriptional activity
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5 under hypoxia following 8 hr of 2-DG treatment compared to untreated GBM cells (Fig. 3E).
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7 Notably, by adding succinate together with 2-DG this reduction did not occur, and addition of
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9 succinate alone doubled HIF-1 α transcriptional activity (Fig. 3E).
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17 *3.5 Hypoxia inhibits the pro-apoptotic effect caused by 2-DG in GBM-derived cells.*

18 We sought to investigate whether these changes of GBM metabolic profile and the observed
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20 reduction of HIF-1 α protein level correlated to block of cell cycle and induction of apoptosis.
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22 It has already been reported that downregulation of HIF-1 α suppresses tumorigenicity of
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24 renal cell carcinoma through induction of apoptosis [41] and attenuates glioma growth in
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26 vivo [42]. Analysis of cell cycle revealed that addition of 2-DG in GBM cells acutely
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28 exposed to high oxygen tension induced a modest but not significant decrease of S phase and
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30 a slight increase of apoptotic cells (sub G1 fraction), but these effects were not observed in
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32 hypoxia (Fig. 4A). Analysis of apoptosis by using annexin-V/propidium iodide staining
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34 (annexin-V/PI) indicated that addition of 2-DG significantly induced early apoptosis
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36 (annexin-V⁺/PI cells) especially when GBM cells were exposed for 24 and 48 hr to high
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38 oxygen tension (Fig. 4B). Oppositely, addition of 2-DG on SVZ-derived cells did not
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40 promote significant cell cycle changes and/or induction of apoptosis (not shown).
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48 Apoptosis often implicates disruption of mitochondrial membrane potential ($\Delta\psi_{mt}$). We found
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50 that addition of 2-DG for 24 and 48 hr caused reduction of $\Delta\psi_{mt}$ especially when cells were
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52 exposed to 20% oxygen (Fig. 4C). Moreover, we found that anti-apoptotic proteins (Bcl-2
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54 and Bcl-XL), involved in mitochondria controlled apoptosis, were down-regulated more
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56 rapidly by addition of 2DG under 20% oxygen, while PARP cleavage, which is a marker of
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1 cells undergoing apoptosis [43], occurred earlier under the same conditions (Fig. 4D).
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3 Importantly, even though decrease of Bcl-2 and Bcl-XL proteins occurred even after 18 hr
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5 after addition of 2-DG, percentage of early apoptotic cells (annexin-V⁺/PI cells) increased
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7 significantly only after 48 hr (Fig. 4B). In normal SVZ-derived cells these apoptosis-related
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9 proteins were not affected at early time points, but only after prolonged 2-DG stimulus
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11 (Suppl. Fig. 2C).

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13 Analysis of p21^{cip1}, which is related to cell cycle arrest and induction of differentiation or to
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15 cell death [44], revealed that GBM cells exposed to 20% oxygen, independently from
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17 addition of 2-DG, had a higher p21^{cip1} level compared to cells maintained under hypoxia,
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19 confirming our previous report [7] (Fig. 4D). Under hypoxia addition of 2-DG slowly up-
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21 regulated p21^{cip1} (Fig. 4D) to level comparable to the one observed at 20% oxygen. In SVZ-
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23 derived cells p21^{cip1} did not change following addition of 2-DG (Suppl. Fig. 2C).
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31 *3.6 2-DG induces neuronal commitment and addition of succinate antagonizes this effect by* 32 33 *reducing GBM differentiation.*

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35 HIF-1 α expression has been related to the maintenance of a more immature phenotype in
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37 ductal breast carcinoma and neuroblastoma [2,3], a phenomenon that has been recently
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39 described also in malignant gliomas [5,7]. As we found that addition of 2-DG within the first
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41 18 hr induced increase of p21^{cip1}, with apoptosis significantly occurring after 48 hr (Fig. 4B-
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43 D), together with HIF-1 α poly-ubiquitylation and degradation even under hypoxia (Fig. 3A),
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45 we evaluated if these events correlated to the acquisition of a more differentiated phenotype
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47 in live remaining GBM cells. Flow cytometric analysis of cell surface marker CD133,
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49 revealed that addition of 2-DG promoted a decrease of CD133⁺ cells percentage, especially at
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51 20% oxygen (Fig. 5A). Normal SVZ-derived cells contained a lower percentage of CD133⁺
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53 cells compared to GBM-derived cells, and addition of 2-DG analogously induced a slight
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1 decrease of CD133⁺ cells (Suppl. Fig. 2D). Importantly, we found that addition of succinate
2 under hypoxia increased CD133⁺ cells in GBM (Fig. 5A), and this did not occur in SVZ-
3 derived cells (Suppl. Fig. 2D). Addition of both 2-DG and succinate slightly prevented 2-DG-
4 mediated decrease of tumor CD133⁺ cells exposed to 20% oxygen (Fig. 5A). Another
5 described GBM stem cell marker is CD90 [45], which we found to be highly expressed in all
6 our GBM cell cultures. Nevertheless, only a slight decrease of CD90⁺ cells was observed
7 after addition of 2-DG and succinate did not reverse this effect (Suppl. Fig. 4).
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9 Immunocytochemical analysis on adherent remaining cells of nestin (Fig. 5B,E), indicative of
10 undifferentiated precursors, glial fibrillary acidic protein (GFAP) (Fig. 5C,E), a marker of
11 astroglia and their progenitors, and β III-tubulin (Fig. 5D,F), expressed in neurons and their
12 committed progenitors, revealed that addition of 2-DG under hypoxia promoted specifically
13 neuronal differentiation (i.e. higher β III-tubulin⁺ cells percentage), rather than glial
14 commitment, with decrease in nestin⁺ cells. In GBM cells acutely exposed to 20% oxygen a
15 higher basal neuronal and glial commitment was visible and under this condition the addition
16 of 2-DG did not further increase differentiation. Opposite to 2-DG effects, addition of
17 succinate raised fraction of nestin⁺ cells (Fig. 5B, E), lowering the percentage of GFAP⁺ and
18 β III-tubulin⁺ cells (Fig. 5C-F). Finally, addition of succinate together with 2-DG prevented 2-
19 DG mediated differentiation of GBM-derived cells (Fig. 5B-D). Altogether these data suggest
20 that addition of 2-DG promotes induction of GBM cell differentiation and lately cell death
21 under 20% oxygen, while maintaining cells at 2% oxygen partially inhibits these effects,
22 preserving tumor stem cells survival, despite HIF-1 α degradation occurs.
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51 **4. Discussion**

52 There have been increasing efforts to characterize the solid tumor microenvironment and to
53 define which signalling molecules and metabolic responses may be disrupted in tumor cells.
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As the most primitive tumor cells share characteristics with non-tumor stem cells [46], the microenvironment that regulates stem cell self-renewal and differentiation may also play a role in the proliferation and fate of tumor cells. In particular, there is evidence that hypoxia plays a key role in normal homeostasis of stem cells [9] and in the initiation, development and aggressiveness of gliomas [47]. Accordingly, our recent report confirmed that intratumoral hypoxia drives GBM stem cells distribution [48]. Hypoxia could be crucial to recruit cancer stem-like cells, deregulating their differentiation [2,3,7] and altering their metabolism, by increasing tumor glycolytic response and reducing mitochondrial metabolism. Particularly, among mitochondrial dehydrogenases, SDH has been found inactive in some solid tumors, and this has been described as a key event leading to the accumulation of intra-cytoplasmic succinate and ROS causative of the “pseudo-hypoxic” effect. This is a major tumor-related hallmark, characterized by the activation of HIF even in the absence of hypoxia [14, 15]. Moreover, recent reports indicate that the gene encoding isocitrate dehydrogenase-1 (IDH1), one of the enzyme of citric acid cycle which is related to HIF-1 pathway, is mutated in 80% of certain types of GBM [49-51], thus linking mitochondrial dysfunction to tumorigenesis.

2-DG, a glucose analogue that reduces intracellular utilization of glucose, inhibits glycolysis, through competitive inhibition of some glycolytic enzymes. 2-DG can affect the growth of some tumors in vivo and it has been shown to be cytotoxic in vitro [52]. In a recent work HIF-1 α has been shown to mediate resistance specifically to glycolytic inhibitors [53], however, the molecular mechanisms by which hypoxia may counteract 2-DG effects in brain tumor has not been so far fully elucidated. A decrease in provision of glucose (from 25 mM to 2,5 mM) and also of oxygen (2%) can be found in the internal perinecrotic/hypoxic region of the solid tumor mass compared to peripheral, relatively well-nourished regions of the tumor [54]. Thus, in this study we set our control maintaining GBM-derived cells at 2%

1 oxygen and in presence of high glucose concentration. We show that inhibiting glycolysis by
2 addition of 2-DG, induces a decrease in cell proliferation and late apoptosis in primary GBM-
3 derived cells acutely exposed to 20% oxygen, while these effects are partially inhibited by
4 hypoxia, condition in which 2-DG addition causes GBM cell differentiation and HIF-1 α level
5 reduction. Importantly, normal SVZ-derived cells are not affected by addition of 2-DG.
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11 Degradation of HIF-1 α by 2-DG has been described in a previous report [37], nevertheless a
12 molecular explanation for this effect has not been so far fully provided. Here we show that 2-
13 DG increases SDH activity, reducing ROS levels and these are reliably causative of the
14 observed HIF-1 α degradation, consequentially to increased PHD2 activity. Indeed, HIF-1 α is
15 known to be chemically stabilized also by hypoxia independent factors, such as ROS [38],
16 CoCl₂, Fe²⁺ [39] and cytoplasmic succinate [14]. Earlier studies have suggested that ROS
17 produced by mitochondria are required for proper induction of HIF-1 α under hypoxic
18 conditions [55, 56]. It has been well established, that Fe²⁺ is an important cofactor for PHDs
19 [39], and importantly H₂O₂, produced by mitochondria, is known to destabilize PHDs
20 activity, either by converting Fe²⁺ in Fe³⁺ or by induction of post-transcriptional
21 modifications of PHD2 [55]. Besides ROS acting as second messengers, impaired SDH
22 activity and consequential intra-cytoplasmic succinate accumulation are associated to HIF-1 α
23 stabilization in several cancer types [14]. We found that addition of succinate reverts 2-DG
24 effects, promoting recovery of HIF-1 α protein level and transcriptional activity (i.e. HRE-
25 luciferase), as well as increase of ROS levels. Besides succinate, PHDs have been shown to
26 be regulated also by other glycolytic and citric acid cycle metabolites, such as pyruvate and
27 oxaloacetate, that could independently modify the oxygen-sensing abilities of cells [57].
28 While we found that PHD2 protein level is raised by addition of 2-DG, *PHD2* transcription is
29 decreased, and this is related to the observed degradation of HIF-1 α , known to control *PHD2*
30 transcription [58]. Additionally, HIF-2 α has been found highly expressed in glioma stem cells
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1 [39], but we did not find change of HIF-2 α protein level following neither stimulus with 2-
2 DG, nor addition of succinate.
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4 Importantly, HIF-1 α is related to maintenance of an undifferentiated cell state and inhibition
5 of cell differentiation [7, 11]. Particularly, HIF-1 α contributes to a differentiation defect in
6 malignant gliomas [5], even following exogenous BMP2 treatment [7, 8], shedding new light
7 on the differentiation therapy of solid tumors by HIF-1 α targeting. Therefore, HIF-1 α has
8 become in recent years an attractive, although challenging, therapeutic target [59]. Here, we
9 found that after addition of 2-DG, a decrease of CD133⁺ and nestin⁺ cells and induction of
10 neuronal differentiation occur under hypoxia, condition in which GBM cells are maintained
11 in a less committed state [7,48]. Oppositely, addition of succinate increases GBM cell
12 undifferentiated state, preventing the pro-differentiating effects evoked by 2-DG.
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15 Previous reports describe that highly de-differentiated and fast-growing tumors compared to
16 slow-growing tumors or non-tumor cells are characterized by faster glycolysis and slower
17 oxidative phosphorylation [60-63]. Here we found that diverse mechanisms may control
18 GBM cell phenotype: on one side addition of 2-DG and raised oxygen tension induce GBM
19 cell commitment and eventually cell death; oppositely to these, succinate and hypoxia
20 promote GBM immature cells maintenance (Fig. 6).
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23 In conclusion, we show that reducing glycolysis in GBM tumor cells destabilizes cell
24 functional and metabolic status, forcing cancer cells into a mitochondrial metabolism, as
25 demonstrated by the activation of PDH and SDH, and promoting GBM cell differentiation.
26 These findings improve the knowledge of GBM tumor intra- and extra-cellular
27 microenvironment and the mechanisms by which GBM cells respond to an alteration of their
28 metabolic state.
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Competing interests

The authors declare that they have no competing interests.

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Figure legends**Figure 1.**

Glycolysis block by 2-DG reduces extracellular lactate and lowers proliferation in GBM-derived cells. (A) Representative pictures of GBM-derived cells (HuTuP01), plated at medium density (49 cells/mm²) and expanded 1 day (18 hr) in 2% or 20% oxygen in the presence of 25 mM glucose or of 25 mM 2-DG (in low glucose medium, containing 2.5 mM glucose). Pictures acquired with an IX50 Olympus inverted microscope; 10X magnification. Bar = 100µm. (B, C) Extracellular lactate measurement following 2-DG addition, comparing 2 different GBM cell cultures (B) and 3 SVZ-derived cell cultures (C), cultured under 2% oxygen or 20% oxygen at the indicated times. ***p < 0.001 above 0 hr time point bars indicate statistically significant differences with all the other time points. (D, E) Cell growth kinetics: GBM cells (D) and SVZ cells (E) were plated at medium density (T0 = 49 cells/mm²); cells were either maintained in glucose (solid line) or in 2-DG (dashed line) for 4, 8 and 24 hr at either 2% (light gray) or transferred to 20% oxygen (dark grey). Final cell counts/mm² were normalized to plating density at T0. Mean of 4 different tumors ± S.E.M., n = 3 for each tumor. Mean of 3 different SVZ cell cultures ± S.E.M., n = 3 for each cell culture. (F) Analysis of metabolic activity by MTT assay; cells were cultured under 2% oxygen or 20% oxygen and 2-DG was added for 18 or 24 hr, values were normalized to cells cultured with glucose at 2% O₂. (G) Intracellular ATP content in GBM cells cultured as described in (F), values normalized to cells cultured with glucose at 2% O₂.

Figure 2.

Block of glycolysis with 2-DG increases SDH and PDH activity. (A) Representative citochemical analysis (HuTuP13) of SDH activity by using NBT reduction methodology. Cells were incubated for 48 hr in presence of glucose, 2-DG or 3-NPA known to irreversibly inactivate SDH (negative control). (B) Bar graph showing mean percentage of SDH⁺ cells

1 (blue-violet cells) counted from 40X magnification pictures (picture area = 0.02 cm²), bar =
2 20 μm. 3 different tumors have been used, n = 2 for each tumor. (C, D) Representative
3 western blot analyses (HuTuP36) of pPDH E1α and total PDH along with β-actin to control
4 for protein loading. (C) Cells were cultured for 0.5, 2, 8 or 18 hr with 2-DG, either left at 2%
5 oxygen or acutely exposed to 20% oxygen; the analysis was confirmed using 3 different
6 tumors, n = 3 for each tumor. (D, E) Histograms showing percentages of cells with
7 intracellular superoxide anion (O₂⁻) measured by using hydroethidine (HE), mean ± S.E.M.
8 from 3 different tumors, n = 2 for each tumor.
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23 **Figure 3.**

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27 2-DG promotes HIF-1α degradation, while succinate promotes recovery of HIF-1α protein
28 level and transcriptional activity in GBM-derived cells. (A) Representative western blot
29 analyses (HuTuP13) of HIF-1α, HIF-2α and PHD2, along with β-actin to control for protein
30 loading. GBM-derived cells were initially expanded in 2%, then 2-DG was added for 0, 1, 2,
31 4, 8, 18 hr, maintaining cells at 2% or exposing them to 20% O₂. The analyses were
32 confirmed using 3 different tumors, n = 3 for each tumor. (B, C) QRT-PCR analyses of *HIF-*
33 *1α* and *PHD2* normalized to *GUSB* and then calibrated to 2% O₂ 0hr (ΔΔCt Method), mean ±
34 S.E.M. comparing 3 different GBM, n = 3 for each tumor. (D) Representative western blot
35 analyses (HuTuP13) of HIF-1α and HIF-2α along with β-actin to control for protein loading,
36 in GBM derived cells cultured for 18 hr with glucose, 2-DG or diethyl-succinate (5 mM),
37 combined as indicated; to confirm the data 3 different tumors have been used, n = 2 for each
38 tumor. (E) HRE-luciferase assay: GBM cells were transiently transfected either with a HRE-
39 firefly luciferase reporter construct or with a mutated HRE version of the same construct to
40 evaluate aspecific effects. Along with these vectors, also a Renilla luciferase vector has been
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transfected in order to normalize luciferase detection. Normalization of the data, expressed in RLU (relative light unit), to the mutated HRE vector was done and then values were calibrated to untreated cells (Control). 2 different GBM have been analyzed, $n = 3$ for each tumor.

Figure 4.

Hypoxia inhibits the pro-apoptotic effect caused by 2-DG in GBM-derived cells. (A) Histogram showing percentages of different cell cycle phases in live gated cells based on BrdU and 7-AAD incorporation. GBM-derived cells were cultured as described in Fig. 1A. Mean of 3 tumors \pm S.E.M., $n = 2$ for each tumor. (B) Annexin-V/Propidium Iodide (Annexin-V/PI) staining of GBM derived cells expanded for 0, 18, 24 or 48 hr in the presence of 2-DG, either at 2% or 20% oxygen. Bar graph represents percentages of early apoptotic cells (annexin-V⁺/PI⁻ cells) and late apoptotic cells (annexin-V⁺/PI⁺ cells); mean of 3 tumors \pm S.E.M., $n = 3$ for each tumor. (C) Analysis of mitochondrial membrane potential by using JC-1: histogram shows % of low $\Delta\psi_{mt}$ in live gated cells (based on physical parameters, side scatter and forward scatter), mean of 2 tumors \pm S.E.M., $n = 3$ for each tumor. (D) Representative western blot analyses (HuTuP15) of Bcl-2, Bcl-XL, PARP, p21^{cip1} along with β -actin to control for protein loading, in GBM cells cultured for 0, 1, 2, 4, 8, 18 hr in presence of 2-DG, either at 2% or 20% oxygen. The analyses were confirmed using 3 different tumors, $n = 3$ for each tumor.

Figure 5.

2-DG induces neuronal commitment and addition of succinate antagonizes this effect by reducing GBM differentiation. (A) Histogram showing percentages of CD133⁺, mean of 4 tumors \pm S.E.M., $n = 3$ for each tumor. (B-D) histograms showing respectively nestin, GFAP

1 and β -III-tubulin quantitations relative to total DAPI⁺ cells. Mean of 3 tumors \pm S.E.M., n = 2
2 for each tumor. (E,F) Representative immunocytochemical images (HuTuP10) of nestin
3 (red)/GFAP (green) (E) and β -III-tubulin (red) (F). GBM cells were plated at medium density
4 (49 cells/mm²), expanded 18 hr in 2% or 20% oxygen in the presence of glucose, 2-DG
5 and/or succinate. 20X magnification, bar = 40 μ m.
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16 **Figure 6**

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19 Summary of the principal effects elicited by 2-DG, succinate and oxygen tension in GBM
20 cells. (Left part) Under hypoxia GBM cells display high anaerobic glycolysis, low PDH (i.e.
21 phosphorylated PDH) and low SDH activity, the latter causative of intra-cytoplasmic
22 accumulation of succinate and consequentially also of ROS. Both ROS and succinate have
23 been described as inhibitors of PHDs activation, causing the stabilization of HIF-1 α . Addition
24 of succinate elicits analogous effects, as shown by HIF-1 α stabilization and increased number
25 of GBM stem cells. (Right part) Addition of 2-DG associate to exposure to 20% O₂ increase
26 PDH activity (i.e. de-phosphorylated PDH) and consequentially SDH activity, thus
27 intracellular succinate and mitochondrial ROS release are reduced, according to literature. As
28 a consequence PHD2 activity increases, HIF-1 α is poly-ubiquitylated and consequentially
29 degraded, which correlates with the acquisition of a more committed phenotype, according to
30 previous works. Under these circumstances GBM cells undergo more rapidly apoptosis.
31 Importantly, all these effects are mitigated by the hypoxia and by addition of succinate, which
32 prevent SDH activity and tumor cell differentiation induced by 2-DG.
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Figure 1

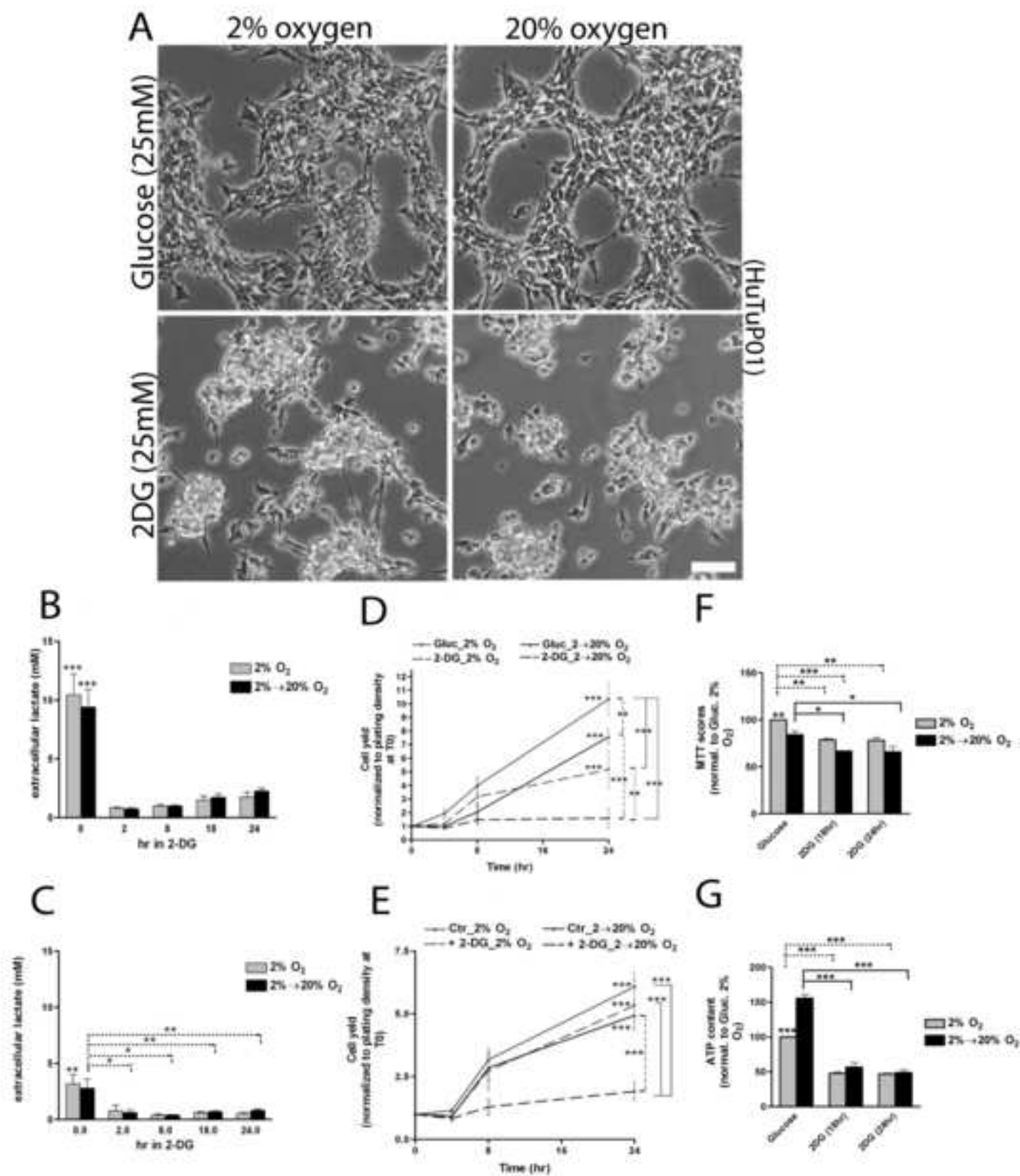


Figure 2

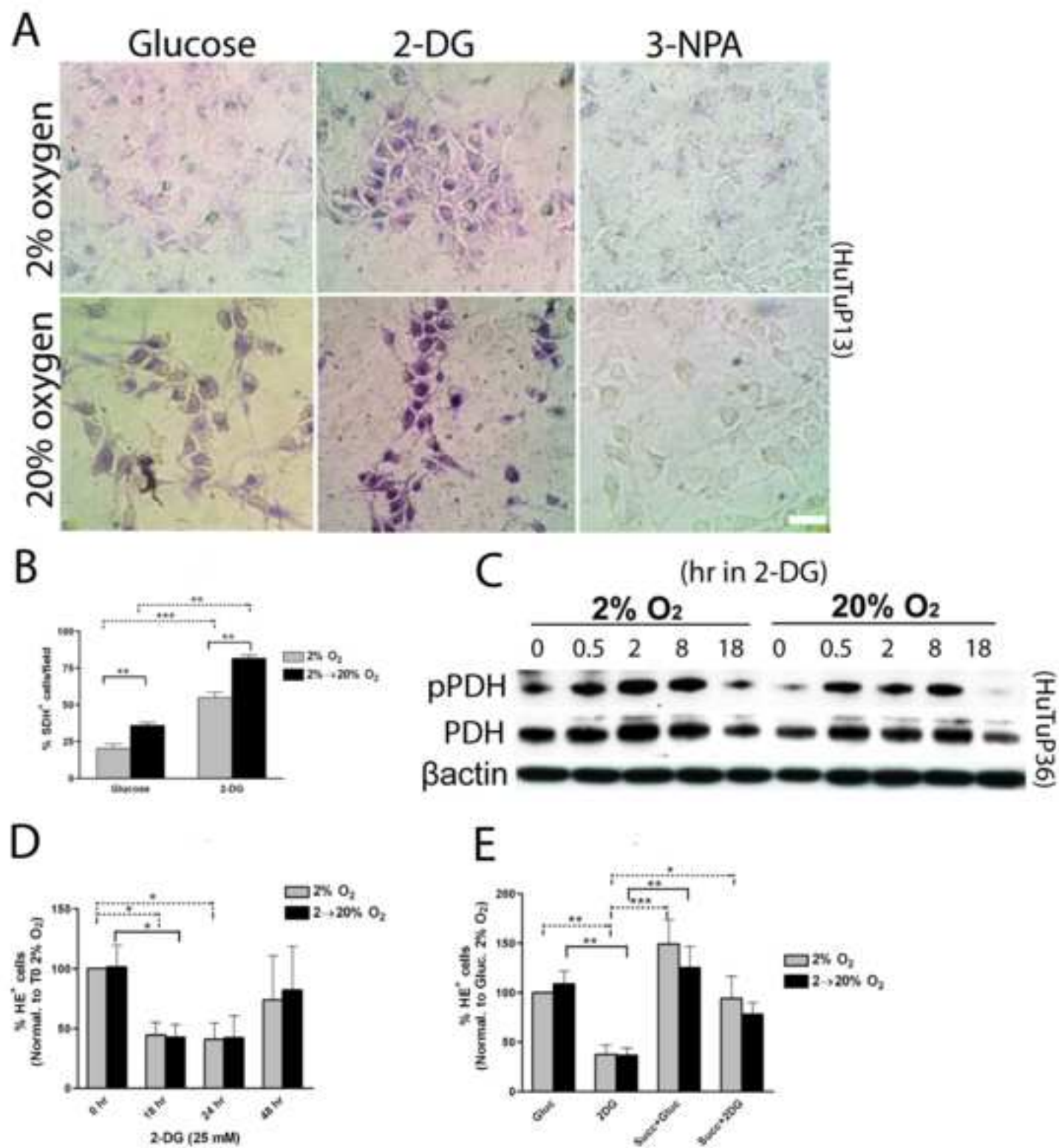


Figure 3

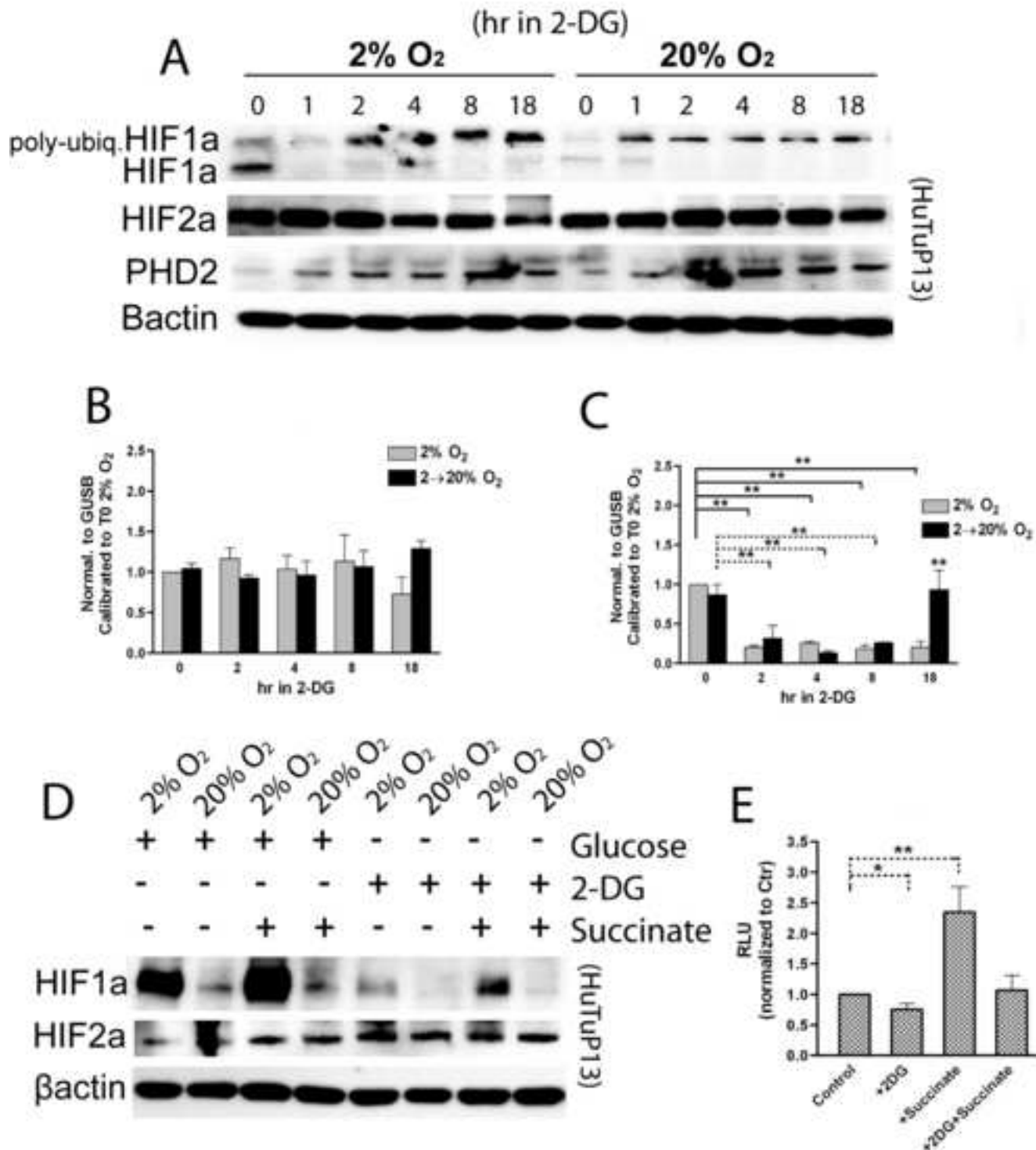


Figure 4

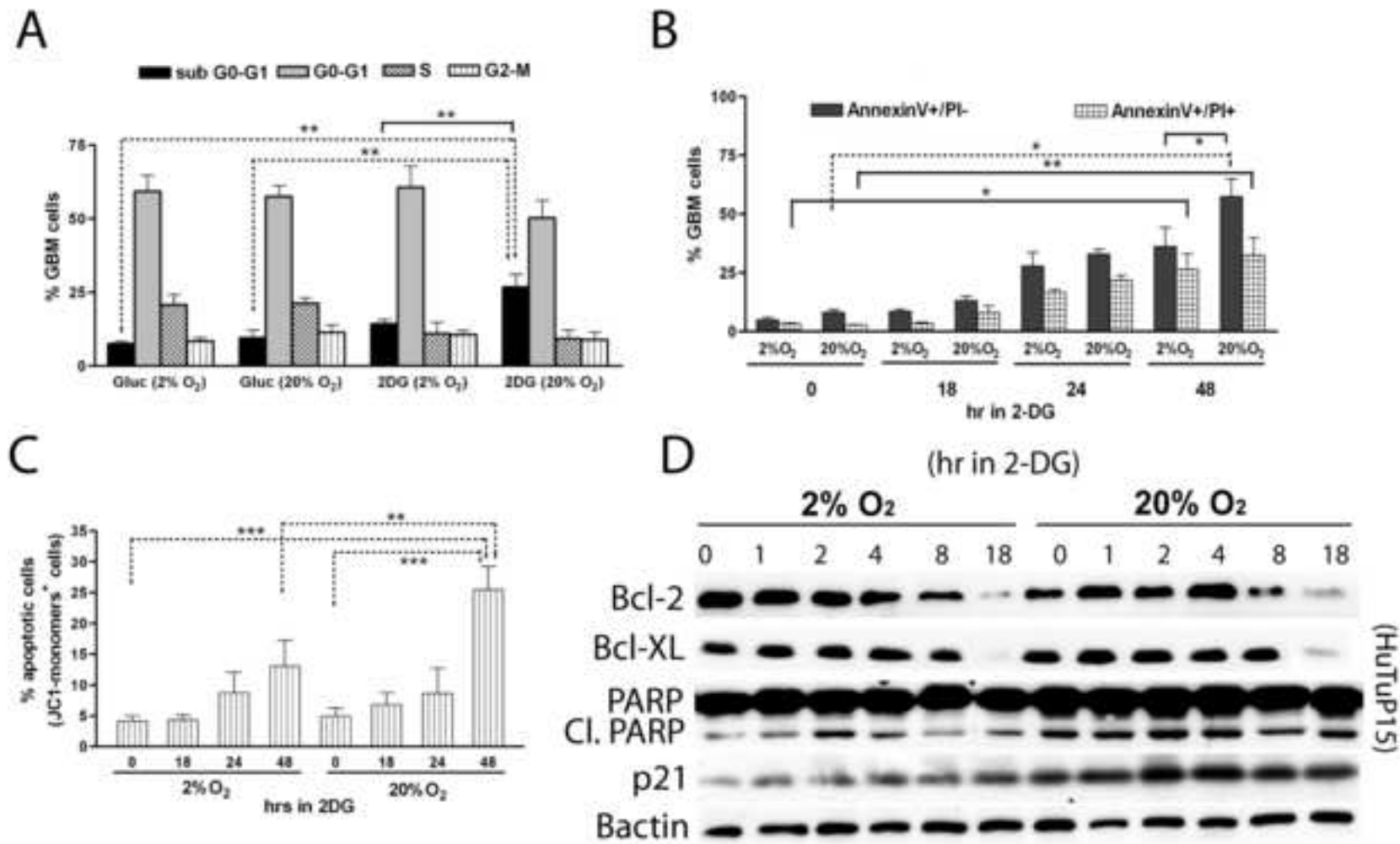


Figure 5

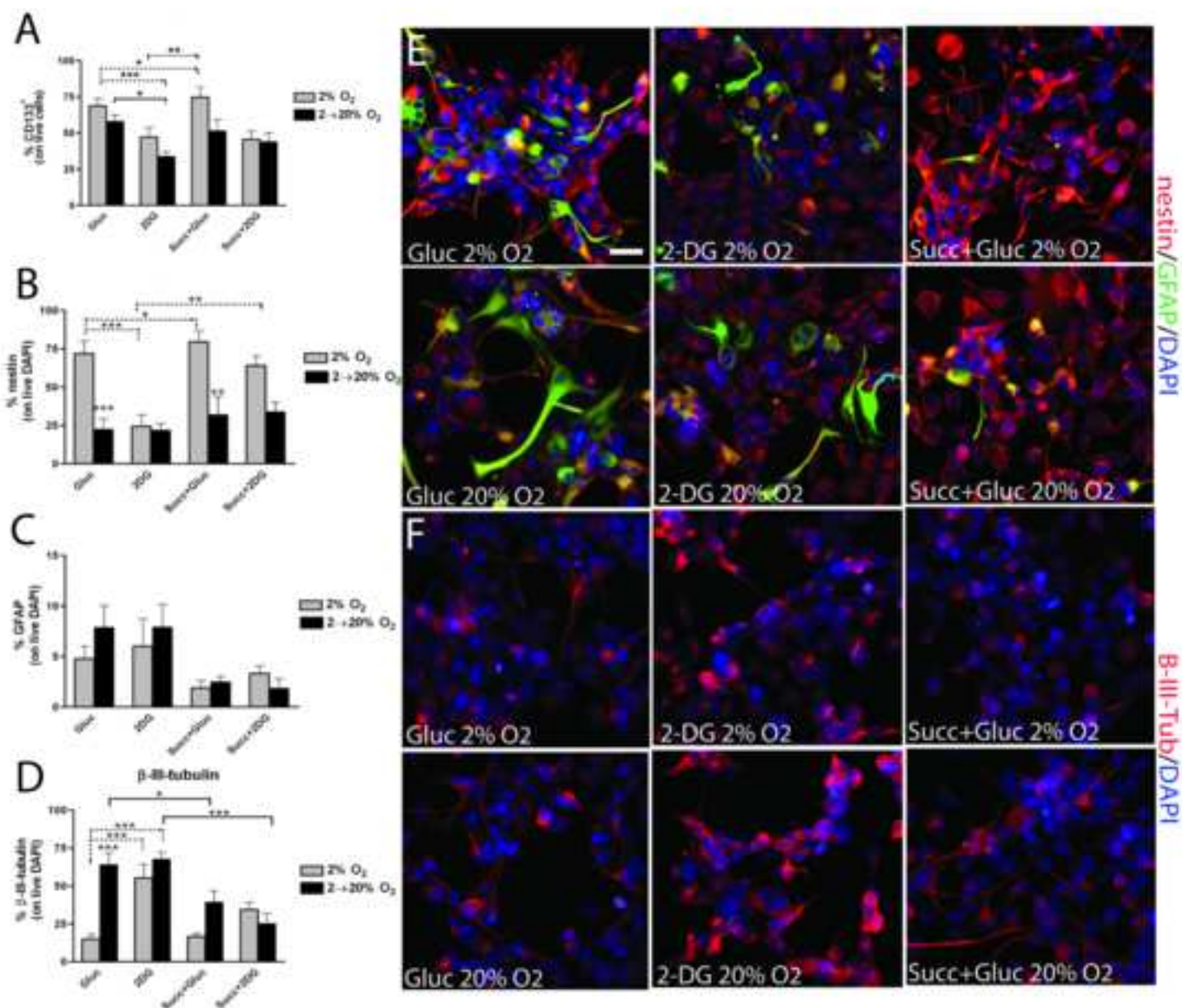
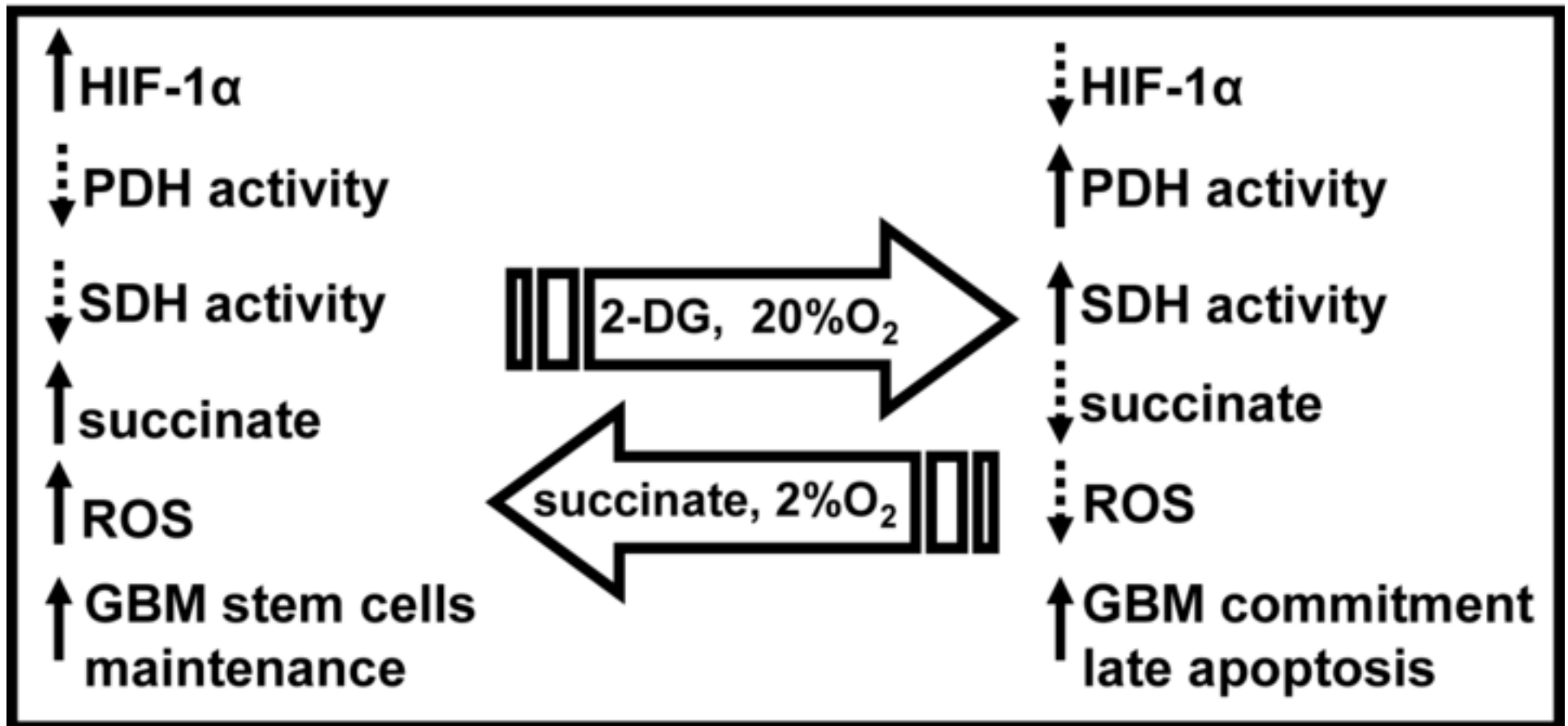


Figure 6



Effects of 2-DG, succinate and oxygen tension in Glioblastoma (GMB) cells

