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Richards R, Jenkinson MD, Haylock BJ, See V. 2016. Cell cycle progression in glioblastoma cells is unaffected by pathophysiological levels of hypoxia. PeerJ 4:e1755 https://doi.org/10.7717/peerj.1755

Cell cycle progression in glioblastoma cells is unaffected by pathophysiological levels of hypoxia

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Hypoxia is associated with the increased malignancy of a broad range of solid tumours; however, whilst very severe hypoxia has been widely shown to induce cell cycle arrest, the impact of pathophysiological hypoxia on tumour cell proliferation is poorly understood. The aim of this study was to investigate the effect of different oxygen levels on glioblastoma (GBM) cell proliferation and survival. GBM is an extremely aggressive brain tumour with a heterogeneous oxygenation pattern. The effect of a range of oxygen tensions on GBM cell lines and primary cells were assessed using flow cytometry. Results indicate that cell cycle distribution and viability are unaffected by long term exposure (up to 4 days) to pathophysiological levels of oxygen (1-8% O_2). Both transient cell cycle arrest and small amounts of cell death could only be detected when cells were exposed to severe hypoxia (0.1% O_2). No significant changes in p21 protein expression levels were detected. These findings reinforce the importance of using physiologically relevant oxygen tensions when investigating tumour hypoxia, and help to explain how solid tumours can be both hypoxic and highly proliferative, as is the case with GBM.

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Cell cycle progression in glioblastoma cells is unaffected by pathophysiological levels of hypoxia

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Abstract

Hypoxia is associated with the increased malignancy of a broad range of solid tumours; however, whilst very severe hypoxia has been widely shown to induce cell cycle arrest, the impact of pathophysiological hypoxia on tumour cell proliferation is poorly understood. The aim of this study was to investigate the effect of different oxygen levels on glioblastoma (GBM) cell proliferation and survival. GBM is an extremely aggressive brain tumour with a heterogeneous oxygenation pattern. The effect of a range of oxygen tensions on GBM cell lines and primary cells were assessed using flow cytometry. Results indicate that cell cycle distribution and viability are unaffected by long term exposure (up to 4 days) to pathophysiological levels of oxygen (1-8% O_2). Both transient cell cycle arrest and small amounts of cell death could only be detected when cells were exposed to severe hypoxia (0.1% O_2). No significant changes in p21 protein expression levels were detected. These findings reinforce the importance of using physiologically relevant oxygen tensions when investigating tumour hypoxia, and help to explain how solid tumours can be both hypoxic and highly proliferative, as is the case with GBM.

Introduction

Tumour growth is dependent on the formation of new blood vessels; however the vasculature within tumours is poorly organised and often shows severe structural abnormalities. This leads to regions of the tumour experiencing a reduced supply of oxygen, known as hypoxia [1-3]. Hypoxia is a fundamentally important hallmark of solid tumours and is associated with tumour progression and poor patient prognosis across a broad range of tumour types[4, 5]. Paradoxically, hypoxia has also been reported to induce cell cycle arrest[6]. This reduction in proliferation could reduce tumour burden, or alternatively it may increase tumour aggressiveness due to the central role of the cell cycle in mediating sensitivity to both chemo- and radiotherapy[7, 8]. To resolve the apparent contradiction between the pro-tumorigenic role of hypoxia and its reported anti-proliferative effects, we performed a systematic investigation into the effect of different oxygen levels on glioblastoma (GBM) cell proliferation and survival.

GBM is the most common primary malignant brain tumour with 3 per 100,000 people diagnosed every year[9]. Despite advances in neurosurgery and radiotherapy over the past 40 years, the prognosis remains poor with an average survival time of just 14 months[10]. GBM is a diffusely infiltrative Grade IV astrocytic tumour, characterised histopathology by brisk mitotic activity, cellular and nuclear atypia, vascular thrombosis, microvascular hyperproliferation and necrosis[11]. As with many solid tumours, GBM is characterised by a heterogeneous pattern of oxygenation. Investigations using oxygen-sensitive electrodes have shown the normal level of oxygen in human brain tissue to be 5 - 8%. In contrast, mean oxygen levels in high-grade gliomas range from 0.75 - 2.76% [12-18]. There is a trend for increased levels of hypoxia in high-grade, as compared to low grade, gliomas and a greater hypoxic tumour volume has been found to correlate directly with a poorer prognosis in GBM[17, 19].

There are a number of mechanisms through which hypoxia promotes tumour malignancy, including resistance to radio- and chemotherapy[20, 21], increased cell migration and invasion[22], reprogramming towards a cancer stem cell (CSC) phenotype and expansion of CSC populations[23-25]. At a cellular level, the master regulator of oxygen homeostasis is hypoxia-inducible factor (HIF), a transcription factor that promotes cell survival in low oxygen conditions by activating the transcription of multiple genes, including those involved in angiogenesis, glycolysis and invasion[26]. HIF-1α expression has been found to correlate with tumour grade in gliomas, with increased expression found in high-grade gliomas[27, 28].

In contrast to other aspects of tumour malignancy, the effects of hypoxia on cell cycle regulation are poorly characterised. It is often stated that hypoxia induces cell cycle arrest, however these observations have been made in severe hypoxia (≤0.1% O_2) or anoxia[6, 29, 30]. Investigations using the 2-nitroimidazole EF5, an agent which forms macromolecular adducts in low-oxygen levels as a result of its reductive metabolism[31], have established that the proportion of severely hypoxic cells in brain tumours is low. Rather, the majority of cells are exposed to moderate hypoxia (> 0.5% O_2)[32]. Research into the effect of more physiologically relevant oxygen tensions on tumour growth is lacking.

The aim of this study was to investigate the effects of physiological (8% O_2), pathophysiological (1% O_2) and severe (0.1% O_2) levels of hypoxia on GBM cell proliferation and survival. We demonstrate that cell cycle progression in GBM cells is unaffected by pathophysiological levels of hypoxia, and that only severe hypoxia is capable of causing transient cell cycle arrest or cell death.

Methods

Cell culture and hypoxic treatment. All reagents were purchased from Life Technologies unless otherwise stated. U87 cells (ATCC, HTB-14), U251 cells (CLS, 300385), and D566 cells (a kind gift from Professor DD Bigner, Duke University Medical Centre, USA) were maintained in MEM supplemented with 1% sodium-pyruvate and 10% foetal bovine serum (FBS). In addition, U251 and D566 cells were supplemented with 1% non-essential amino acids (NEAA). HeLa cells (ECACC, 93021013) were maintained in MEM plus 10% FBS and 1% NEAA. All cells were maintained at 37 °C in 5% CO_2. Prior to treatment, cells were counted using a Bio-Rad TC20 automated cell counter and seeded at a density of 1 x 10^5 cells in 6 cm tissue culture dishes (Corning). Cells exposed to 1% O_2 were incubated in a Don Whitley H35 Hypoxystation, whilst cells exposed to 8% and 0.1% O_2 were incubated in a New Brunswick Galaxy 48R hypoxic incubator. A media change was performed after 48 h.

Tumour dissection and primary culture. Samples of primary GBM tumours were received from patients undergoing craniotomy and resection, who had given informed written consent to donate their tissue to the Walton Research Tissue Bank, Walton Centre NHS Foundation Trust, which has full approval of the National Research Ethics Service (11/WNo03/2). Primary cell culture was carried out in accordance with the approved guidelines. Tumour samples were transported in MEM plus 1% penicillin-streptomycin (pen-strep), mechanically dissected, and transferred into dissociation medium (10% trypsin 10X and 1% DNase [Sigma] in MEM plus 1% pen-strep). Samples were incubated for 15-30 min at 37 °C and triturated every 5 min. The trypsin reaction was stopped by adding growth medium (MEM plus 20% FBS, 1% sodium-

pyruvate and 1% pen-strep); cells were then centrifuged for 5 min at 3000 rpm, resuspended in growth medium and seeded into a 75 cm^2 tissue culture flask.

Flow cytometry. For the viability analyses, adherent cells were washed with phosphate buffered saline (PBS), trypsinised and suspended, together with the floating fraction of cells, in Hank's balanced saline solution (HBSS). The cell suspension was transferred to a 96 well plate, stained with FITC Annexin V (1:500) and incubated for 15 min in the dark at room temperature. Propidium iodide (PI) was added at a final concentration of 4 µg/mL immediately prior to analyses. Samples were analysed using a Guava EasyCyte Flow Cytometer (Millipore) and cell viability was established using GuavaSoft software (Millipore). For cell cycle analyses, cells were washed with PBS, trypsinised and resuspended in PBS. PI and ribonuclease A were added at a final concentration of 10 µg/mL and Triton X-100 at 0.1%. Cells were analysed by flow cytometry and the percentage of cells in G_0/G_1, S and G_2/M phases was established using ModFit LT (Verity Software House).

Western blotting. 30-40 µg protein was resolved on a 10% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane and probed with primary antibodies against HIF-1α (BD Biosciences 610959), β-actin (Abcam ab8226), (all at 1:1000) and p21 (Santa Cruz sc-396, 1:500) at 4° C overnight. Membranes were incubated with either an anti-mouse (1:5000) or anti-rabbit (1:3000) horseradish peroxidase-linked secondary antibody (Cell Signalling) for 1 h at room temperature. Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) was used according to the manufacturer's instructions prior to detection using a G:BOX gel imaging system (Syngene, UK).

Real time reverse transcription polymerase chain reaction (qRT-PCR). RNA was extracted using a HP RNA isolation kit (Roche) according to manufacturer's protocols. Reverse transcription of 1 µg mRNA to cDNA was carried out using a SuperScript VILO cDNA synthesis kit according to the manufacturer's instructions. Real time polymerase chain reaction (qPCR) was performed in triplicate using LightCycler® 480 SYBR Green I Master (Roche) according to the manufacturer's instructions, 2 µl cDNA and 0.5 µM of each primer. Primers used were as follows: cyclophilin A forward: GCTTTGGGTCCAGGAATGG, reverse: GTTGTCCACAGTCAGCAATGGT; p21 forward: AGCTGCCGAAGTCAGTTCCTT, reverse: GTTCTGACATGGCGCCTCCT; p27 forward: TCCGGCTAACTCTGAGGACA, reverse: GAAGAATCGTCGGTTGCAGG; E2F1 forward: CCGCCATCCAGGAAAAGGTG, reverse: GTGATGTCATAGATGCGCC. qPCR was performed using a LightCycler 480 (Roche); for parameters see Table S1. Results were analysed using LightCycler 480 software (version 1.5.0.39). The values for target genes were normalised to cyclophillin A and expressed as fold change from control values (20% O_2).

Statistical Analyses. Statistical significance was determined by ANOVA using SPSS statistical software (IBM).

Results

Pathophysiological hypoxia has minimal effects on GBM cell proliferation and survival, however long-term exposure to severe hypoxia results in cell death. In order to investigate the effect of atmospheric (20% O_2), physiological (8% O_2) and pathophysiological (1% O_2)

oxygen tensions on cell survival, three GBM cell lines (D566, U87 and U251 cells) were exposed to different oxygen levels for up to 4 days. Cell proliferation and survival were measured using Annexin V/PI staining and cell counting using flow cytometry. D566 and U87 cell proliferation was not significantly affected, although there was a trend for lower proliferation in 1% O_2 in D566 cells (Fig. 1A). U251 cells were more sensitive and displayed a reduction in cell number following 96 h exposure to 1% O_2 (Fig. 1A). The viability of all three cell lines was not affected (Fig. 1B). These results suggest that, although GBM cell lines may vary slightly in their sensitivity to changes in oxygen levels, pathophysiological levels of hypoxia do not significantly affect cell proliferation or survival.

We investigated whether severe hypoxia (0.1% O_2) could have a more pronounced effect on cell fate. In both D566 and U251 cells a significant reduction in cell number was evident after 48 h exposure to 0.1% O_2 (Fig. 2A), whilst U87 cell proliferation remained relatively unaffected until 96 h, at which point a significant decrease in cell number was observed. A reduction in cell number can be due either to a reduced proliferation rate or an increase in cell death. The latter was the case for U251 and U87 cells, which showed a significant decrease in viability after 48 h exposure to 0.1% O_2 (Fig. 2B). In both U251 cells (Fig. 2C) and U87 cells (Fig. S1) cell death was predominantly due to necrosis In contrast, the viability of D566 cells was unaffected by exposure to 0.1% O_2, suggesting that the reduction in D566 cell number may be due to cell cycle arrest.

Cell cycle distribution is unaltered by pathophysiological hypoxia, whilst severe hypoxia induces a transient cell cycle arrest in D566 cells only. As a more direct assessment of the effect of hypoxia on cell proliferation, cells were exposed to 0.1%, 1% or 20% O_2 prior to cell

cycle analyses by flow cytometry. There was no difference in cell cycle distribution between cells exposed to 1% O_2 and 20% O_2 in any of the cell lines tested (Fig. 3A). However after 48 h exposure to 0.1% O_2 D566 cells displayed a 13% increase ($p = .002$) in the proportion of cells in G_1 phase. Interestingly, this cell cycle arrest was only transient and was lost in chronic hypoxic conditions ($\geq$ 72 h) (Fig. 3A; Fig. S2). In U87 and U251 cells there were no significant differences in cell cycle distribution between cells exposed to 0.1% and 20% O_2 at any time point (Fig. 3A). In order to confirm that resistance to hypoxia induced cell cycle arrest is not an acquired characteristic of cell lines maintained in culture, we conducted the same experiment with primary GBM cells isolated from tumour samples obtained at resection (designated GBM03 and GBM04). As with the U87 and U251 cell lines we saw no evidence of cell cycle arrest in 1% or 0.1% O_2 (Fig. 3B). These results demonstrate that only severe hypoxia has the potential to induce cell cycle arrest in GBM cells and, furthermore, this arrest is only transient.

As GBM cells are known to harbour mutations in a number of genes involved in cell cycle control[33], we verified that these cells were capable of undergoing cell cycle arrest by exposing them to 10 μM etoposide for 24 h. All three cell lines displayed a profound S phase arrest (Fig. S3), confirming that the absence of cell cycle arrest in hypoxia was not due to a general lack of capacity of these cells to arrest. To investigate whether the observed transient cell cycle arrest in 0.1% O_2 was specific to GBM cells or common to other cancer cells, we exposed HeLa cells to 0.1%, 1% and 20% O_2 for up to 72 h. As with the GBM cells, cell cycle distribution was unaffected by exposure to 1% O_2. Whilst an increase in cells in G_1 phase was observed following 48 h exposure to 0.1% O_2, this increase was not significant, and, as observed previously in D566 cells, cell cycle distribution returned to normal after 72 h (Fig. 3B).

GBM cells respond to hypoxia with an increase in HIF-1α protein expression; however p21 expression is not significantly altered. We confirmed the ability of GBM cells to respond to hypoxia by measuring HIF-1α stabilisation using western blot (Fig. 4A). In all three cell lines, HIF-1α was stabilised under hypoxic conditions with increased protein levels in 0.1% compared to 1% O_2 (Fig. 4B). To further confirm the absence of a significant and long lasting effect of hypoxia on cell cycle, mRNA levels of the cyclin-dependent kinase inhibitors p21, p27 and the well described transcription factor involved in G_1/S transition, E2F1, were measured by qRT-PCR following up to 96 h exposure to 0.1% O_2 (Fig. 4C). No significant changes in p27 or E2F1 expression were observed in any of the cell lines, and no changes in p21 expression were observed in U87 or U251 cells. D566 cells showed an increase in p21 expression at 24 h, with expression levels peaking at 48 h and decreasing after 72 h, consistent with the temporary G_1 phase accumulation observed in D566 cells exposed to 0.1% O_2 (Fig. 3A). Western blotting was used to investigate whether this transient increase in p21 mRNA was translated at the protein level. Only a very small increase in protein was detected after 24h exposure to 0.1% O_2 (Fig. 4D). Furthermore, this increase was negligible compared to the amount of p21 detected following treatment with etoposide, a potent inducer of cell cycle arrest (see Fig. S3).

Discussion

Our results clearly demonstrate that GBM cells survive and proliferate following chronic exposure to 1% O_2, which is typical of the oxygen levels reported in brain tumours. We have shown that only severe hypoxia (0.1% O_2) is capable of inducing GBM cell cycle arrest and, furthermore, this arrest is only transient. Previous research has indicated that hypoxia causes cell cycle arrest; however in these experiments cells were exposed to anoxia or, at best, severe

hypoxia, which is not representative of the oxygen levels found within tumours [6, 29, 30, 34]. Furthermore, previous investigations have used short-term exposures (≤ 48 h) and so the transiency of the cellular response was not detected.

Whilst none of the cell lines tested showed any changes to cell cycle distribution following exposure to pathophysiological levels of oxygen (1% O_2), U251 cells displayed a reduction in cell number after 96 h. This suggests that that in certain cell lines progression through each phase of the cell cycle may be slowed following long-term exposure to hypoxia. A G_1 cell cycle arrest was only observed in one cell line (D566 cells) following exposure to severe hypoxia. This transient arrest was associated with a parallel increase in p21 mRNA; however, these changes were not statistically significant and not translated at the protein level. No changes in p27 or E2F1 gene expression were detected. Previous investigations have demonstrated an association between cell cycle arrest induced by severe hypoxia and p21 and/or p27 expression, although there is controversy as to whether these proteins are essential[34, 35-37]. Goda *et al*. reported that HIF-1α-mediated induction of p21 and p27 is essential for hypoxia induced G_1 cell cycle arrest[36]. Gardner *et al*. also reported that hypoxia induces p27; however in contrast to Goda *et al.* they found no involvement of HIF-1α in hypoxia induced G_1 arrest[34]. The reason for these discrepancies is unclear but may be due to differences in cell lines and methodologies.

When we compared cell proliferation under physiological oxygen levels (8% O_2, 'physioxia') to standard cell culture conditions (20% O_2), no changes were detected. These findings contrast with previous research which has suggested a pro-proliferative role for physioxia; for example, fibroblasts display an elevated growth rate when cultured in 3% O_2. They also display a delayed onset of replicative senescence; a consequence of the reduction in levels of oxidative damage accumulated under these conditions[38, 39]. Increasing evidence

suggests that, although only present in small amounts above 1% O_2, HIF1-α plays a pro-proliferative role in physiological oxygen conditions. Neural progenitor cells display increased proliferation in 10% O_2 in an effect mediated by HIF-1α, and the moderate induction of HIF-1α in 5% O_2 has been shown to promote proliferation of both cancer and non-cancer cell lines, suggesting an important role in normal cell physiology[40, 41].

After 48 h exposure to severe hypoxia (0.1% O_2) we found that U251 and U87 cells underwent cell death. This observation is in line with the well-established finding that cell survival *in vitro* is compromised by exposure to extreme hypoxia[42]. In GBM the most severe regions of hypoxia, as indicated by high EF5 binding, have been found to be adjacent to regions of necrosis[32]. One of the defining characteristics of GBM is pseudopalisading necrosis: an area of necrosis that often arises following a vaso-occlusive event, surrounded by a dense arrangement of cells organised into parallel rows[43]. The surviving cells on the periphery have been shown to express high levels of HIF-1α and matrix metalloproteinases and are thought to be actively migrating outward towards a more favourable environment[28, 44, 45]. As such, regions of necrosis are associated with higher grade tumours and a poorer prognosis[46].

One mechanism by which hypoxia is thought to increase resistance to chemotherapy is by induction of cell cycle arrest. As traditional chemotherapeutics target rapidly diving cells this is thought to allow hypoxic cells to escape cell death[21]. Our research suggests that only a small proportion of cells are exposed to hypoxia severe enough to cause cell cycle arrest, and of these cells a proportion will undergo cell death. It is therefore likely that other mechanisms play a more important role in hypoxia-mediated chemoresistance; for example the increased expression of the ABCB1 and p-glycoprotein drug transporters[47]. Tumour hypoxia is also associated with increased expression of O6-methylguanine-DNA-methyltransferase (MGMT), a DNA repair

256 protein involved in resistance to temozolomide[48]. Decreased diffusion of drugs to hypoxic areas
257 due to increased distance from blood vessels is also likely to play a major role[49].

258 In conclusion, our results indicate that GBM cell proliferation and survival is unaffected
259 by pathophysiological levels of hypoxia, and that even severe hypoxia has only minimal effects
260 on cell cycle. As such, the proliferation of the majority of cells in high-grade gliomas will not be
261 affected by the reduced oxygen supply that is characteristic of these tumours. These findings
262 suggest the absence of a significant role for hypoxia in cell cycle regulation, and demonstrate the
263 importance of using physiologically relevant oxygen concentrations before drawing conclusions
264 about the effects of hypoxia on cell fate.

Acknowledgements

We thank Dr Carol Walker for her role in co-ordinating access to tumour samples through the Walton Research Tissue Bank. We also extend our thanks to Mr Andrew Brodbelt of the Walton Centre NHS Foundation Trust for obtaining the samples.

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Exposure to physiological and pathophysiologicallevels of hypoxia has minimal effects on GBM cell proliferation and survival.

GBM cell lines were exposed to 1%, 8% and 20% O_2 for the indicated time points. (A). Cell proliferation was assessed by cell counting using flow cytometry (B). Cell survival was assessed by Annexin/PI staining and flow cytometry. Figure shows mean of three experiments + SEM. * $p < 0.05$ ** $p < 0.01$

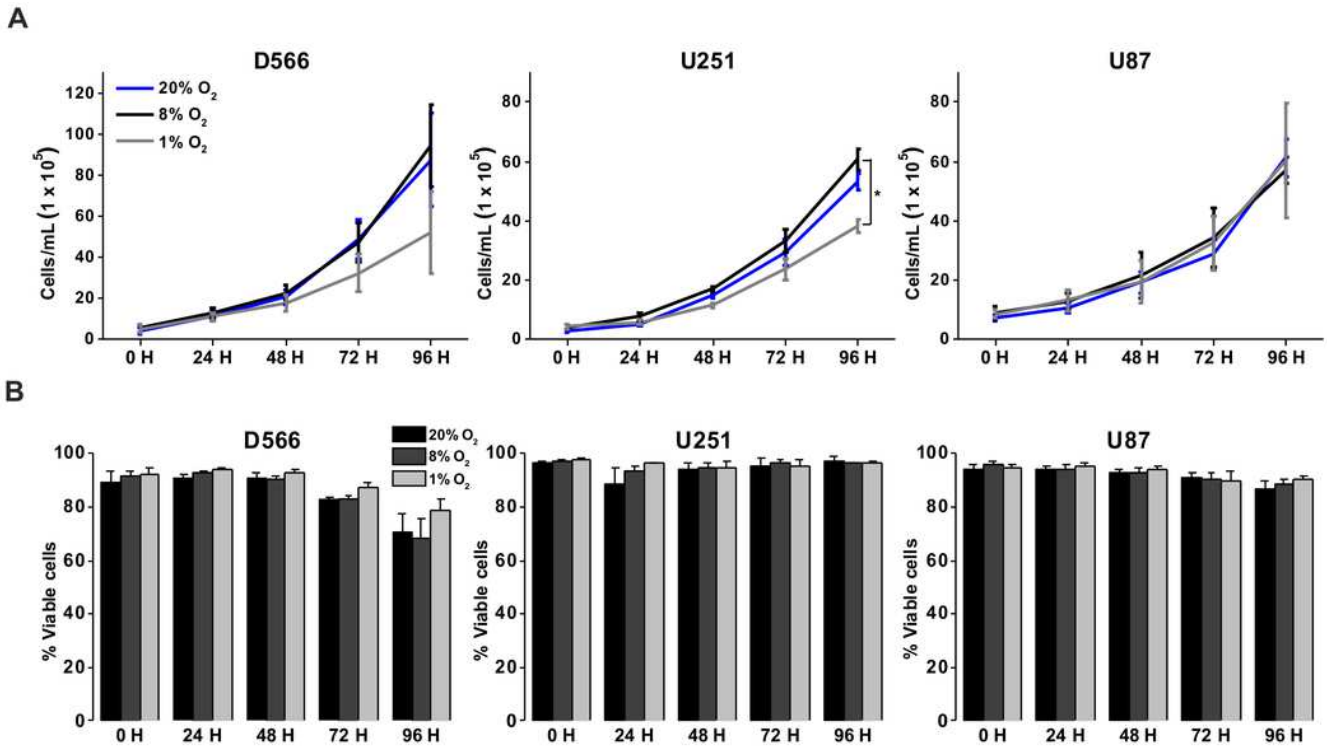


Figure 1

2

Long-term exposure to severe hypoxia causes GBMcell death.

GBM cell lines were exposed to 0.1% and 20% O_2 for the indicated time points. (A). Cell proliferation was assessed by cell counting using flow cytometry (B). Cell survival was assessed by Annexin/PI staining and flow cytometry. (C). Representative example of U251 flow cytometry profile. Figure shows mean of three experiments + SEM. * $p < 0.05$ ** $p < 0.01$

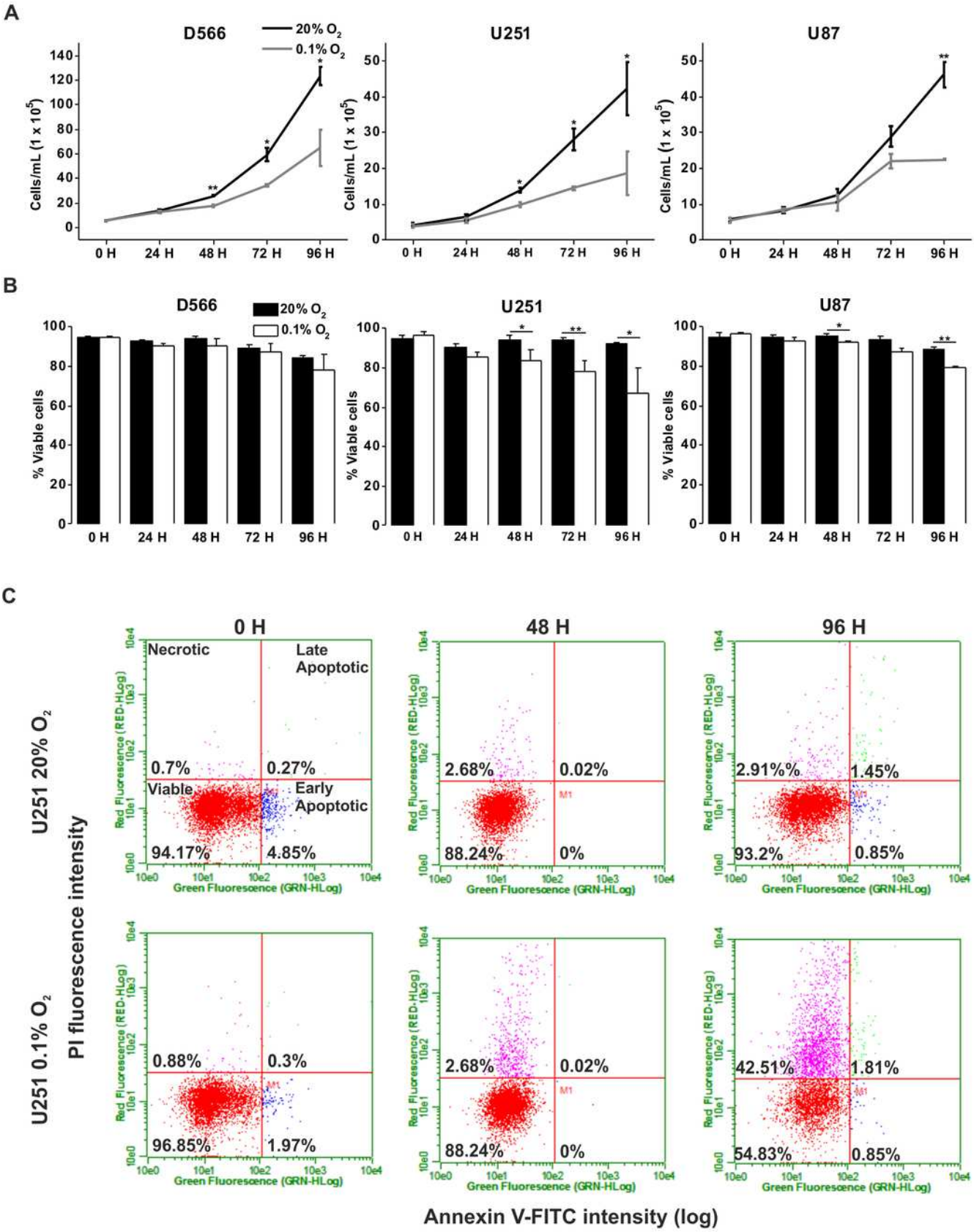

A
D566
U251
U87
20% O2
0.1% O2
Cells/mL (1 x 10^5)
0 H
24 H
48 H
72 H
96 H
B
% Viable cells
C
0 H
48 H
96 H
U251 20% O2
U251 0.1% O2
PI fluorescence intensity
Annexin V-FITC intensity (log)
Red Fluorescence (RED-HLog)
Green Fluorescence (GRN-HLog)
Necrotic
Late Apoptotic
Viable
Early Apoptotic
0.7%
0.27%
94.17%
4.85%
2.68%
0.02%
88.24%
0%
2.91%%
1.45%
93.2%
0.85%
0.88%
0.3%
96.85%
1.97%
42.51%
1.81%
54.83%
0.85%

Figure 2

3

Severe hypoxia causes a transient G_1 phase arrest in D566 cells only.

Cells were exposed to 0.1%, 1% and 20% O_2 for the indicated time points and cell cycle distribution was assessed using flow cytometry. (A). Figure shows mean of three experiments + SEM, with the exception of primary cell lines GBM03 (*N* = 1), and GBM04 (*N* = 2) where figure shows mean + SD. * $p < 0.05$, ** $p < 0.01$.

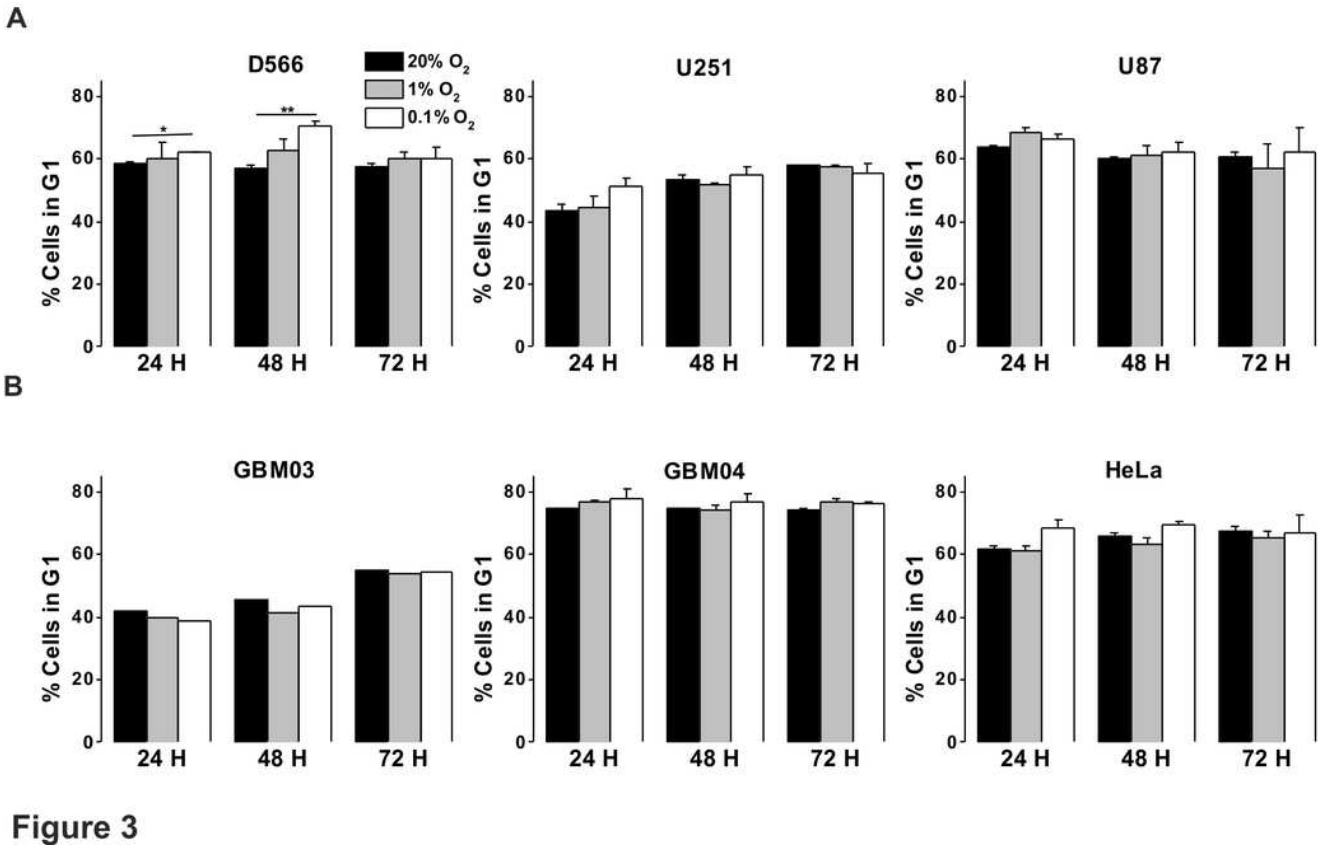


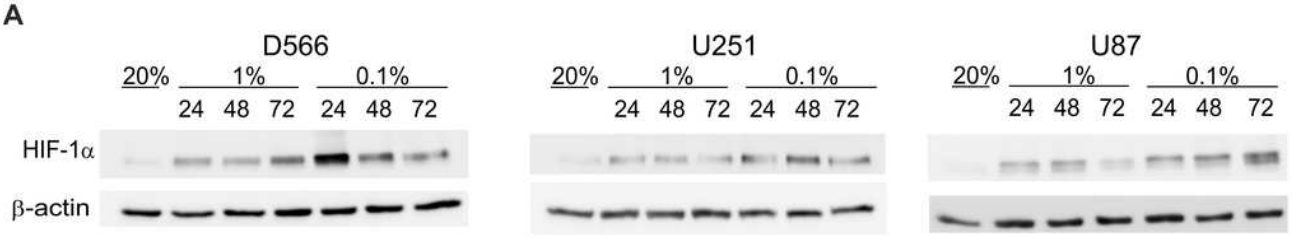
Figure 3

4

Hypoxia increases HIF-1α but does not change p21 protein expression.

Protein expression was examined by western blot following exposure to 20%, 1% or 0.1% O_2 for the indicated time points. Blots are representative examples taken from three experiments. (A). Western blots of HIF-1α protein expression. (B). Densitometry of HIF-1α protein expression shown in (A). (C). qRT-PCR was used to assess mRNA levels of p21, p27 and E2F1 following exposure to 0.1% O_2. Values were normalised to cyclophilin A and expressed as fold change from 20% O_2. Figure shows mean of three experiments + SEM. (D). Western blots of p21 protein expression. Cell exposed to the chemotherapeutic etoposide (Etop) were included as a control. The densitometry analysis, measured as the mean background subtracted integrated density for three experiments + SEM, has been plotted.

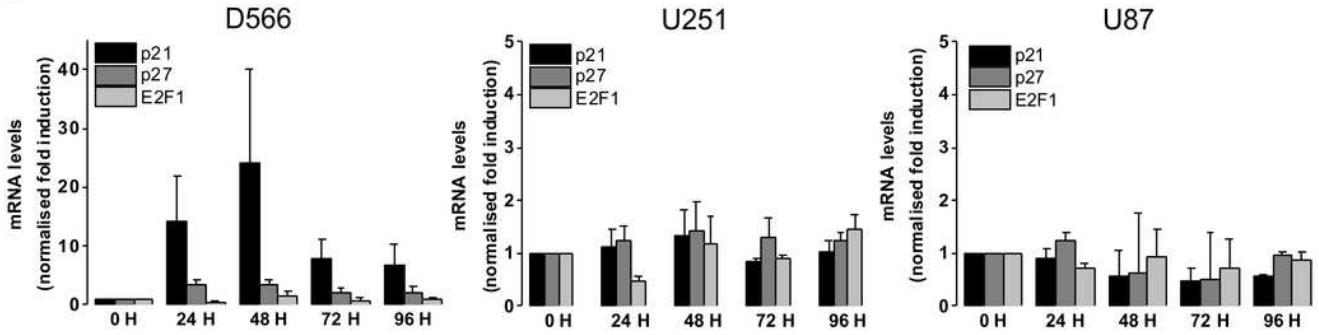
A



B

	1% O_2			0.1% O_2		
	24	48	72	24	48	72
D566	7.98	5.76	7.56	21.71	9.48	6.71
U251	3.17	3.15	2.42	5.68	7.38	5.06
U87	4.05	5.14	2.37	4.62	8.24	10.62

C



D

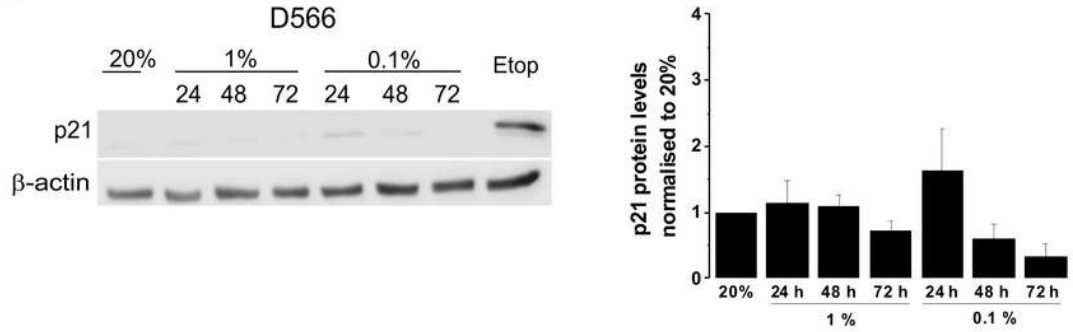


Figure 4