Hypoxia differentially upregulates the expression of embryonic, fetal and adult hemoglobin in human glioblastoma cells

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Abstract. Hemoglobin is produced mainly in erythroid cells. However, it has been reported in non-erythroid cells of human and rodents. We have shown previously that neuroglobin, cytoglobin and hemoglobin are expressed in human glioblastoma multiforme (GBM) cells. We sought to determine whether hemoglobin expression is upregulated by hypoxia, and whether its expression is restricted to the cancer stem cell populations in different GBM cell lines or GBM brain tumor initiating cells (BTICs). Flow cytometry, magnetic cell sorting and qRT-PCR were used to examine the hypoxic upregulation of hemoglobins as well as erythropoietin (EPO) and erythropoietin receptor (EPOR) in GBM cell lines (M006x, M059J, M059K, U87R and U87T) and GBM-BTICs. The data showed significantly increased expression in globins (α, β, γ, δ, ζ and ε), EPO and EPOR mRNA levels under hypoxia. Globin expression is not limited to the stem cell populations or GBM-BTICs but is a property of the entire GBM population. We assumed that the total expression of mRNA of different normalized globins (α, β, γ, δ, ζ and ε) at different time-points for the same cell line is 100%. Under aerobic conditions, ε globin was predominantly expressed, and then decreased gradually with increasing time in hypoxia. This was coupled to a concomitant increase in α and γ globins. Our findings suggest that hypoxic upregulation of hemoglobin expression in GBM cells may be a part of a repertoire of active defence and adaptation mechanisms enabling these cells to acquire resistance to aggressive multimodality treatments of chemotherapy and radiotherapy. New therapeutic strategies to interfere with hemoglobin expression or function in GBM cells are required.

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Introduction

Globin, a basic life protein, has been found in organisms from all kingdoms of life (1). Ancestral globin first appeared 4,000 million years ago (2). The vertebrate globin family consists of four different globins; hemoglobin, myoglobin, neuroglobin (Ngb) and cytoglobin (Cygb), with hemoglobin being the most well studied globin in terms of structure, function and evolution (3). Until recently, it has been thought that vertebrate hemoglobin is expressed only in enucleated red blood cells, the erythrocytes. However, others have reported α and β globin expression in a wide variety of non-erythroid cells, including rodent brain (neurons of the cortex, hippocampus and cerebellum but not astrocytes and oligodendrocytes) (4), embryonic and adult mouse brain neurons (5) and mesencephalic dopaminergic neurons and glial cells in human, mouse and rat (6). Globin production has also been reported in tissues other than brain including embryonic and adult mouse endometrium (7), mouse macrophages (8) and eye lens (9), rat mesangial cells (10), alveolar epithelial type II cells of both rat (11) and human (12); and human breast cancer cells (13).

Adult human hemoglobin is heterotetrameric protein consisting of two α and β polypeptides globin chains, with each globin molecule containing a hydrophobic pocket which non-covalently binds an iron-protoporphyrin IX molecule (14). Hemoglobin expression progresses successively from embryonic [Gower 1 ($\zeta_2\varepsilon_2$), Gower 2 ($\alpha_2\varepsilon_2$) and Portland 1 ($\zeta_2\gamma_2$)] to fetal [Hb F($\alpha_2\gamma_2$)] and then adult [Hb A ($\alpha_2\beta_2$), 97%; Hb A2 ($\alpha_2\delta_2$), <3%] hemoglobin (15). Vertebrate hemoglobin has been shown to not only function as a carrier protein of O_2 and CO_2 (12), but also it generates, transports NO or scavenges NO and its metabolic derivatives (16,17). Other potential functions have been reported including antioxidant and superoxide anion and H_2O_2 scavenging properties (10,18), protecting cells against nitrosative and oxidative stress (10,19).

In vivo and *in vitro* studies have shown that hypoxia increases erythropoiesis through an increase of endogenous erythropoietin, produced mainly in the fetal liver and adult kidney (20) or exogenously when added to hematopoietic progenitor cell culture (21). Similarly, neuronal expression of α and β globin mRNA is increased in EPO-transgenic or EPO-injected mice (4), as well as in normal mice following

stimulation of EPO production (22,23). Upregulation of α and β globin has been reported in retina damaged by hypoxia in hypertensive eye disease and in glaucoma affected eyes (24). Hypoxia-induced erythropoietin signally affected cell survival in the neuronal population.

Glioblastoma multiforme (GBM) is the most common and the most aggressive tumor among gliomas, it constitutes about 50-60% of all astrocytomas and 12-15% of all intracranial neoplasms with a median survival rate of about one year (25,26). Hypoxic regions, hypoxia-induced necrosis and neovascularisation are diagnostic features of GBM (27) where poor survival outcome is associated with increased levels of tumor hypoxia (28). Cancer cells that survive in hypoxic microenvironment are resistant to ionizing radiation and certain chemotherapeutic agents (29). Taken all together and the ability of GBM cells to infiltrate surrounding normal tissues (30) makes curative treatment by surgery, radiation and chemotherapies difficult, if not impossible.

We have previously reported that Ngb, Cygb and hemoglobins are expressed in human GBM cell lines (31-34) as well as human primary tumors, including brain tumors (32). In GBM cell lines, expression of Cygb and Ngb was significantly upregulated when cells were exposed to physiologically relevant levels of hypoxia simulating hypoxic tumor microenviroment (31,32). In this study, we examined whether hemoglobins α, β, γ, δ, ζ and ε are upregulated in human GBM cell lines, and whether their expression is restricted to the cancer stem cell populations in different GBM cell lines or GBM-brain tumor stem cells (BTICs) or is a property common to the entire GBM cell population.

Materials and methods

Cell lines and in vitro culture condition. The origin and characterization of the GBM cell lines have been published previously: the M059J (ATCC no. CRL2366, Manassas, VA, USA) is radio-sensitive and hypoxia-sensitive; M059K (ATCC no. CRL-2365) is radio-resistant and hypoxia-tolerant and M006x cell lines is hypoxia-tolerant (35-38). The U87T (non-invasive) and U87R (invasive) cell lines are established GBM cell lines (39) and were kindly provided by Dr Donna Senger (University of Calgary, Calgary, AB, Canada). All cells were maintained as monolayer cultures in DMEM/F12 media supplemented with 10% fetal calf serum and 1 mM L-glutamine in a humidified atmosphere of 5% CO_2 in air at 37˚C. All tissue culture supplies were purchased from Gibco (Carlsbad, CA, USA).

Generation of hypoxia in vitro. To examine the effect of hypoxia on globin proteins and mRNA expressions measured by western blot analysis and qRT-PCR experiments, respectively. A de-gassing manifold (40) was used to generate hypoxia. Exponential phase cells (~$2x10^5$) were seeded onto 60-mm glass plates and then incubated under standard laboratory culture conditions (5% CO_2 in air) for 4 days. The medium was then replenished and the plates were transferred to aluminum chambers from which the air was evacuated and then replaced with 5% CO_2/balance N_2 until an O_2 tension of 0.6% was achieved. The sealed, air-tight aluminum chambers were then incubated at 37˚C for 6-48 h. The aluminum chambers were unsealed at the end of each incubation interval, the tissue culture plates removed, and then total RNA and cell proteins were isolated.

Table I. TaqMan® Gene expression assays used in qRT-PCR to quantify different globins mRNA.

Gene name	TaqMan® gene expression assay ID
Erythropoietin	Hs00171267_ml
Erythropoietin receptor	Hs00181092_ml
Hemoglobin, α1	Hs00361191_g1
Hemoglobin, β	Hs00747223_g1
Hemoglobin, γG	Hs00361131_g1
Hemoglobin, δ	Hs00426283_m1
Hemoglobin, ζ	Hs00744391_s1
Hemoglobin, ε1	Hs00362216_m1

RNA extraction and reverse transcription. RNeasy mini kit and RNeasy micro kit (both from Qiagen, Valencia, CA, USA) were used to isolate total RNA from GBM cultured cell lines and sorted $CD133^+$ GBM cells, respectively. Reverse transcription (RT) was carried out with 0.1-1 μg total RNA per 20 μl reaction volume using QuantiTect reverse transcription kit (Qiagen). Total RNA isolated from different human GBM-BTICs, labelled 1-5, was kindly provided by Dr Samuel Weiss and Dr Gregory Cairncross (Brain Tumor Stem Cell Core Facility, University of Calgary) (41).

Quantitative real-time reverse transcription-PCR. Quantitative real-time PCR (qRT-PCR) analysis was carried out with a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan fast universal PCR Master mix and validated TaqMan® Gene Expression Assays (Applied Biosystems) for human erythropoietin, erythropoietin receptor and α, β, γ, δ, ζ and ε globin genes (Table I). Human 18S rRNA gene (part no. 4333760T, Applied Biosystems) was used as endogenous control. Amplification data were analyzed with SDS RQ Manager 1.2 software (Applied Biosystems). Fold change in globin genes expression normalized to endogenous control gene (18S rRNA) and relative to normoxic baseline was quantified using $2^{-\Delta\Delta CT}$ ($2^{-\Delta CT\ ([\text{hypoxic sample-endogenous control}]\ \Delta CT\ [\text{normoxic sample-endogenous control}])}$). Fold change of normalized globin genes were calculated as $2^{-\Delta CT\ (\text{sample-endogenous control})} \times 10^6$. To compare the percentages of expression of different normalized globins at different time-points for the same cell line, the fold change expression of individual gene was divided by total fold changes of normalized α, β, γ, δ, ζ and ε globin mRNA.

Flow cytometry and magnetic cell sorting. CD133 was used as marker to screen GBM cell lines (M006x, M059J, M059K, U87R and U87T) for the side population of putative cancer stem cells. Primary cell cultures were washed, resuspended in MACS-BSA stock solution (130-091-376) diluted 1:20 with autMACS rinsing solution (130-091-222) and filtered through

pre-separation filters (130-041-407, all from Miltenyi Biotec, Bergisch Gladbach, Germany). Cells (10^7) were incubated with 20 μl of FcR blocking reagent (130-095-901, Miltenyi Biotec) and 10 μl of the CD133/2(293C3)-PE antibody (130-090-853, Miltenyi Biotec), mixed well and refrigerated for 10 min in the dark. Cells were washed and analysed by flow cytometry (FACSCalibur, BD Biosciences, NJ, USA) for $CD133^+$ stained cells. To isolate $CD133^+$ cells, M006x cells were sorted by both flow cytometry (BD FACSCAria™, BD Biosciences) and a magnetic cell sorting separator (Miltenyi Biotec). In flow cytometry cell sorting experiments, cells were stained with the same antibody used for screening of $CD133^+$ cells. While in magnetic cell sorting, cells were magnetically labelled using a CD133 cell isolation kit (130-050-801, Miltenyi Biotec), separated on MACS MS column (130-041-301, Miltenyi Biotec) attached to Mini MACS separator with column adaptor (Miltenyi Biotec).

Statistics. Data from four replicate experiments were expressed as mean ± SE. Statistical analyses were performed using SigmaPlot 11 software (Systat Software Inc, Chicago, IL, USA). Differences between groups were compared using one-way ANOVA or ANOVA on ranks (Kruskal-Wallis) based on the normality and equal variance tests. To determine exactly which groups are different and the size of the difference, multiple comparisons versus control group were carried out using Bonferroni t-test and Dunnett's or Dunn's test for one-way ANOVA and ANOVA on ranks (Kruskal-Wallis), respectively, as post hoc tests. The all pairwise multiple comparison procedure was used to compare GBM cell lines, M006x-$CD133^+$ and $CD133^-$ cells, and GBM-BTICs for their expression of α, β, γ and ε globin mRNA.

Results

Quantification of globin mRNA expression in GBM cell lines in vitro. GBM cell lines showed increased expression in globin mRNA levels when cultured under hypoxia (0.6% O_2) over a 48 h time period (Fig. 1). α globin mRNA was increased significantly (M006x, M059J, U87R and U87T at 48 h, $P<0.05$; M059K at 24 and 48 h, $P<0.05$). β globin mRNA was increased significantly (M059J and U87R at 48 h, $P<0.05$; U87T at 24 and 48 h, $P<0.05$), while no significant change was observed in M006x and M059K cells. γ globin mRNA was increased significantly at 24 and 48 h (M059J and M059K cells, $P<0.05$) and at 48 h (M006x, U87R and U87T cells; $P<0.05$). δ globin mRNA was increased significantly in M059K (at 24 and 48 h, $P<0.05$) and in M006x, M059J, U87R and U87T cells (at 48 h, $P<0.05$). ζ globin mRNA was upregulated significantly at 24 and 48 h (M059J cells, $P<0.05$), at 24 h (M059K cells, $P<0.05$), and at 48 h (U87R cells, $P<0.05$), while no significant change was observed in M006x and U87T. ε globin mRNA was increased significantly in U87T (at 48 h, $P<0.05$), whereas it was significantly decreased in U87R cells (at 24 h, $P<0.05$). Other cell lines showed modest increase (M006x, M059J, M059K, U87R and U87T cells) at different time-points of hypoxia.

Despite the highest expression of basal ε globin mRNA compared with other globins in all GBM cell lines, its relative fold increase in response to hypoxia was the lowest in comparison to other globins. The order of relative increases in different globin mRNA after hypoxia is proposed as follows: $\alpha > \gamma > \delta > \varepsilon > \beta > \zeta$ globin. Contrary to the unique characteristics of different cell lines (e.g., M006x, hypoxia-tolerant; M059k, hypoxia-tolerant and radio-resistant; M059J, hypoxia-sensitive and radio-sensitive; U87R, invasive; U87T, non-invasive), their significant relative changes in different globins expression were not cell line specific.

Quantification of EPO and EPOR mRNA in GBM cell lines in vitro. In agreement with others (42,43), under aerobic condition, EPO and EPOR mRNA were detected in all GBM cell lines (Fig. 2). Furthermore, under hypoxia (0.6% O_2), four of five cell lines showed significant increase in EPO mRNA expression (M059J, U87R and U87T at 48 h, $P<0.05$; M059K at 24 h, $P<0.05$) (Fig. 2A), while no significant change was observed in M006x cells. Similarly, EPOR mRNA expression was increased significantly by hypoxia (Fig. 2B) in four of five cell lines (M059J, M059K, U87R and U87T at 48 h, $P<0.05$). No significant change was observed in M006x cells.

Percentage of fold change expressions of normalized globin mRNA within different time-points for the same cell line. We assumed that the total expression of different normalized globin mRNA (α, β, γ, δ, ζ and ε) at different time-points for the same cell line is 100%. Comparing percentages of individual globin expression to total globin mRNA among different time-points in GBM cell lines revealed several interesting observations. Under normoxic conditions, ε globin was predominately expressed (~30-97%) within all cell lines when compared to the total globins expression. Under hypoxic conditions and with increasing time of hypoxia exposure, there was gradual decrease of ε globin expression in all cell lines accompanied by concomitant increase in α and γ globin expression and no obvious change in expression of β, δ and ζ globin (Fig. 3).

Expression of CD133 cancer stem cell marker by GBM cell lines. Flow cytometry analysis of GBM cell lines immuno-stained with CD133 (a cancer stem cell marker) as well as the sorted $CD133^+$ M006x cells showed that the percentage of $CD133^+$ cells ranged from 0.02% (U87T) to 0.88% (M059J) of the entire cell population (Fig. 4). Previous study by others using immune-staining of either GBM tumors or GBM cell lines with CD133 antibody showed that the percentage of $CD133^+$ cells varies widely with reported ranges of 0.2-13.9% (44), 0.3-25.1% (45), 10.2-69.7% (46) and 0.5-10% (47).

Expression of α, β, γ and ε globin mRNA in GBM-BTSCs and $CD133^+$ and $CD133^-$ M006x cell fractions under aerobic conditions. Due to low basal expression levels of globins in GBM cell lines, we examined globin expression in human GBM-BTICs established from primary tumors, in established GBM cell lines and in $CD133^+$ and $CD133^-$ sorted GBM cells. However, statistical analysis of all pairwise comparison showed that there was no significant enrichment of α, β, γ and ε globin mRNA expression in GBM-BTICs, in the $CD133^+$ and $CD133^-$ sorted GBM cells, and in established GBM cell lines (Fig. 5). This may suggest that low levels of

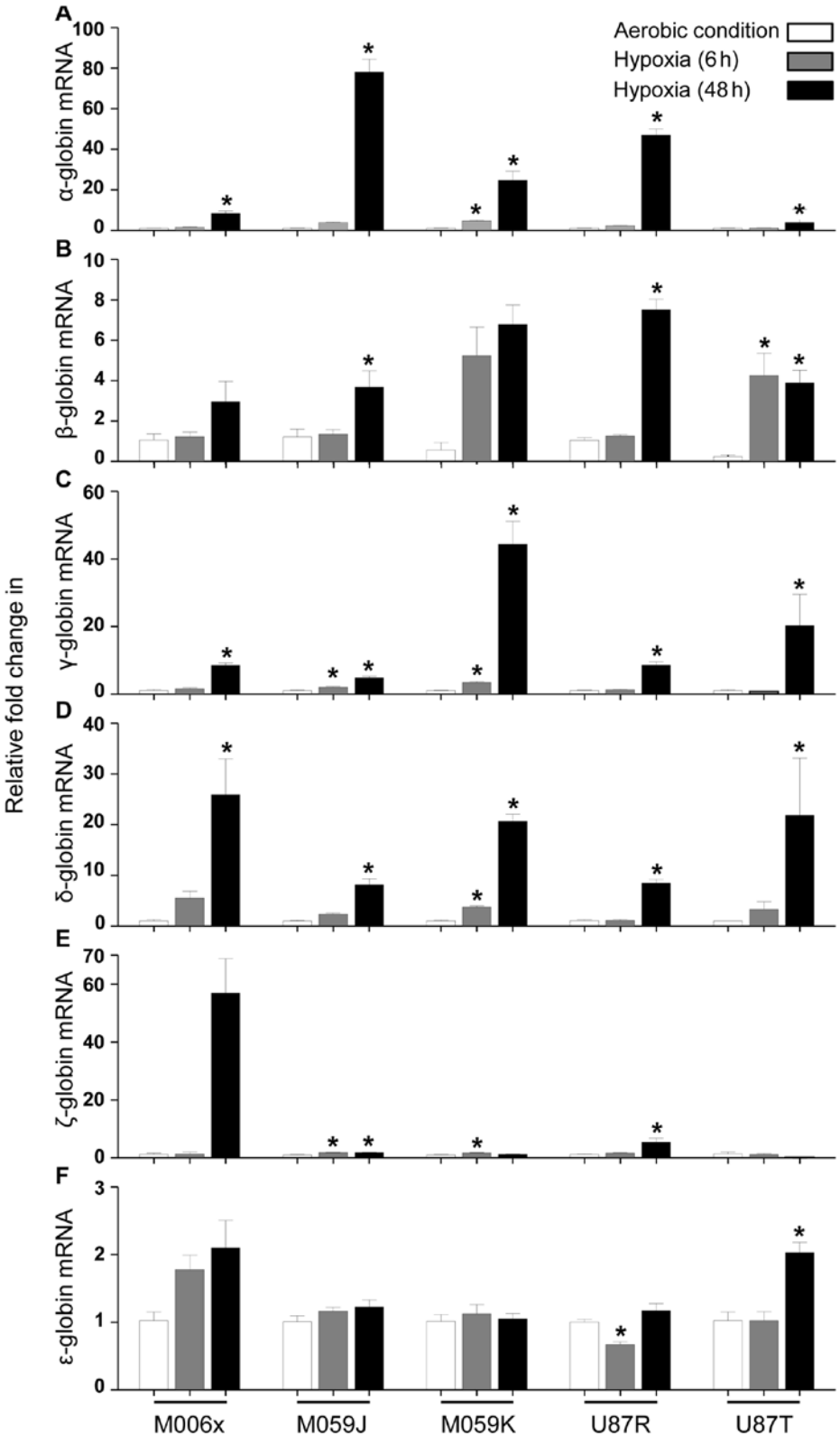

Figure 1. Transcript expression of (A) α-, (B) β-, (C) γ-, (D) δ-, (E) ζ- and (F) ε-globin in human GBM cell lines (M006x, M059J, M059K, U87R and U87T). Expression of mRNA was assessed by qRT-PCR after exposure to hypoxia (0.6% O_2) for 0, 6 and 48 h. Data are expressed as fold increase relative to aerobic control (n=4). *P<0.05 (ANOVA).

globin mRNA are a property of the entire GBM cell population and do not reflect the presence within the cell lines of a small population of putative cancer stem cells with high levels of globin expression.

Discussion

We have previously reported that neuroglobin, cytoglobin and hemoglobin are expressed in human GBM cells (31,32,34). In

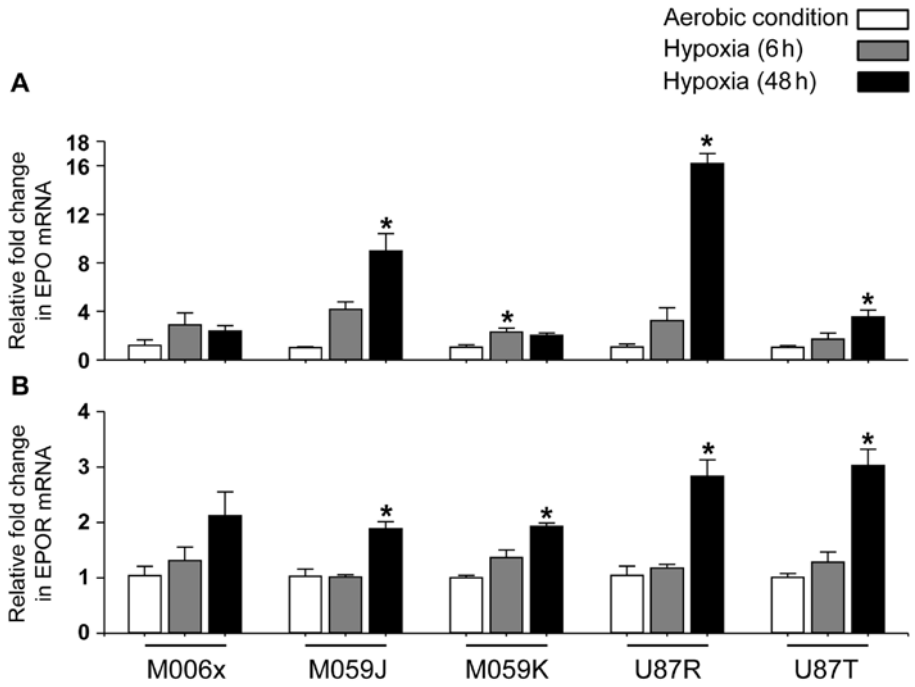

Figure 2. Transcript expression of (A) erythropoietin and (B) erythropoietin receptor in human GBM cell lines (M006x, M059J, M059K, U87R and U87T). Expression of mRNA was assessed by qRT-PCR after exposure to hypoxia (0.6% O_2) for 0, 6 and 48 h. Data were expressed as fold increase relative to aerobic control (n=4). *P<0.05 (ANOVA).

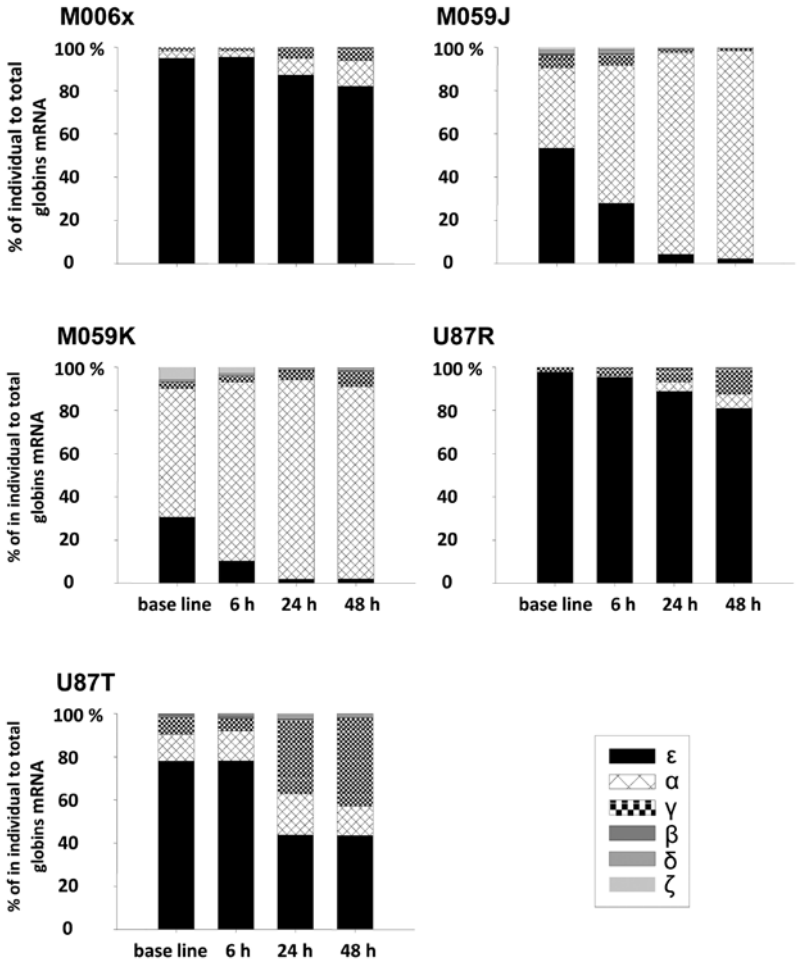

Figure 3. Percentage of individual normalized expression of α-, β-, γ-, δ-, ζ- and ε- to total-globin mRNA in human GBM cells (M006x, M059J, M059K, U87R and U87T). Expression of mRNA was assessed by qRT-PCR after exposure to hypoxia (0.6% O_2) for 0, 6, 24 and 48 h.

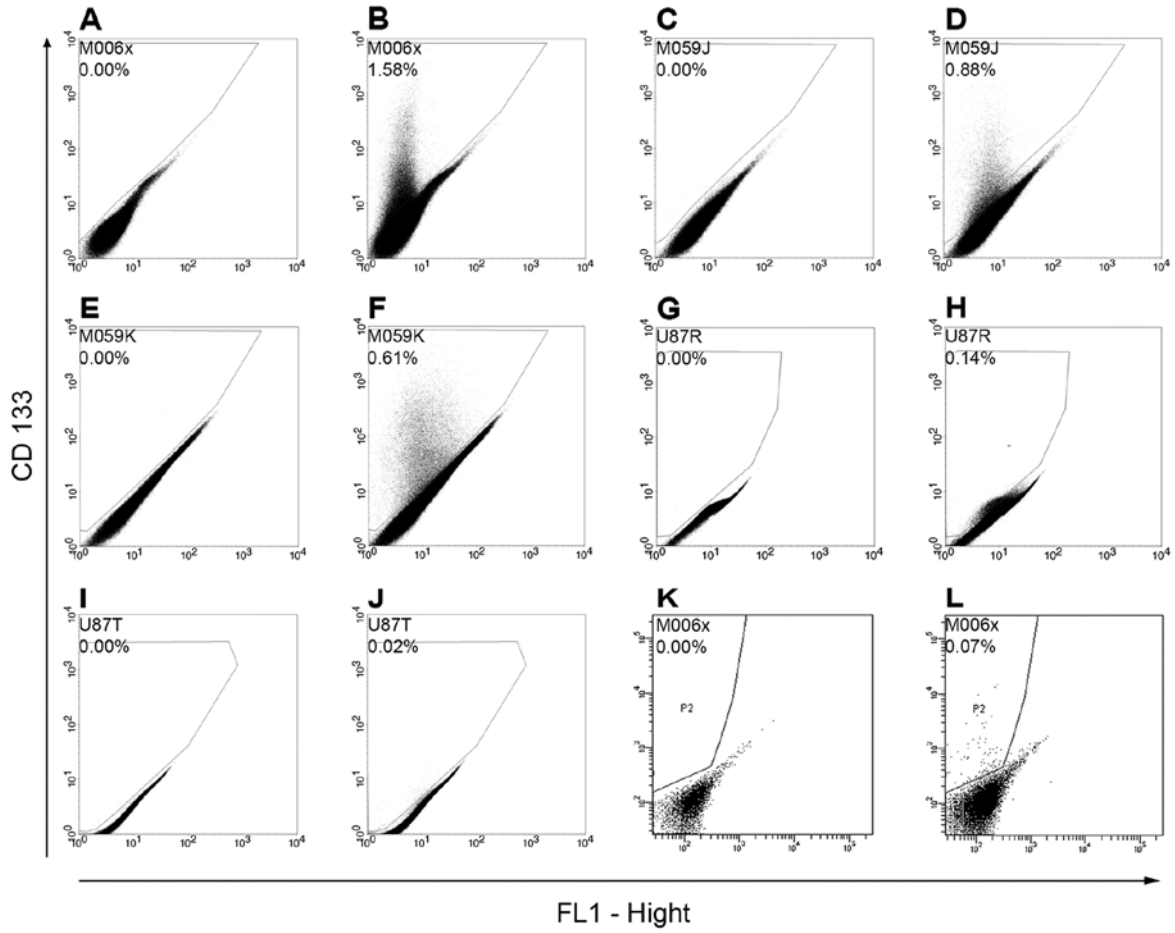

Figure 4. Expression of CD133$^+$ stem cell marker by GBM cell lines. GBM cell lines (M006x, M059J, M059K, U87R and U87T) were immuno-stained with human anti-CD133$^+$ and analyzed by flow cytometry (dotplots B, D, F, H and J) in comparison to their unstained negative control (dotplots A, C, E, G and I). M006x was stained with human anti-CD133$^+$ and sorted by flow cytometry (dotplot K) in comparison to their unstained sorted cells (dotplot L).

this study, using qRT-PCR, flow cytometry and magnetic cell sorting, we have shown significant upregulation in globins (α, β, γ, δ, ζ and ε) mRNA levels in different GBM cells. Hypoxic or ischemic upregulation of α and β globins has been reported in rodent neurons, but not in astrocytes and oligodendrocytes (4,48), whereas their expression in A9 dopaminergic neurons, a subpopulation of cortical and hippocampal astrocytes and in mature oligodentrocytes has been reported (6). The ability of glioblastoma cells to co-express both glial and neuronal markers (49) as a result of their heterogenic nature (50), partially may explain neuroglobin expression in GBM cells (31) as well as hemoglobin (34). The primary functions of erythroid hemoglobin are to bind and transport O_2 and CO_2 (12). In addition, it has been reported to bind CO, to scavenge and release NO (51) and protect the cells against nitrosative and oxidative stress (19,52). On the other hand, the expression and upregulation of neuronal hemoglobin has been linked to regulation of O_2 hemostasis and facilitation of O_2 uptake by neurons in cerebral ischemia (48) and neuronal hypoxia (4), respectively, and in neuronal survival and function (53).

Neuronal expression of α and β globin mRNA is increased in EPO-transgenic or EPO-injected mice as well as by hypoxia in mice via stimulation of EPO production (4,22,23). EPO and EPOR expression and hypoxic upregulation has been previously reported in gliomas (42,54-56). In this study, EPO and EPOR were detected in all GBM cell lines studied. When GBM cells were cultured under hypoxic conditions that simulate *in vivo* O_2 concentrations found in hypoxic regions of human tumors (57,58), EPO and EPOR were significantly increased in four of five GBM cell lines, with no differential expressions of either EPO or EPOR in regards to characteristic features related to hypoxia, radiation or tissue invasion in GBM cells. EPO and EPOR, the principal regulators of erythropoiesis, are inversely correlated with O_2 availability (59,60). In concordance with their functions in non-malignant and non-erythroid tissues (60,61), the EPO and EPOR reported roles in gliomas are to regulate tumor growth (62), promote cell survival (63), invasiveness and survival against chemotherapeutic agents (e.g., cisplatin and temozolomide) and radiation (42,64). Our findings of hypoxic induction of different globins (α, β, γ, δ, ζ and ε) in gliomas may suggest a new function of EPO in brain tumors analogous to its main role in promotion of erythropoiesis.

Interestingly, we observed a switch in globin expression depending on O_2 level. Hemoglobin ε accounted for 30-97% of total globin expression in GBM cell lines under aerobic conditions. However, when cells were exposed to hypoxia, ε globin levels gradually declined with increasing time of hypoxia and this was coupled to an increase in expression of α and

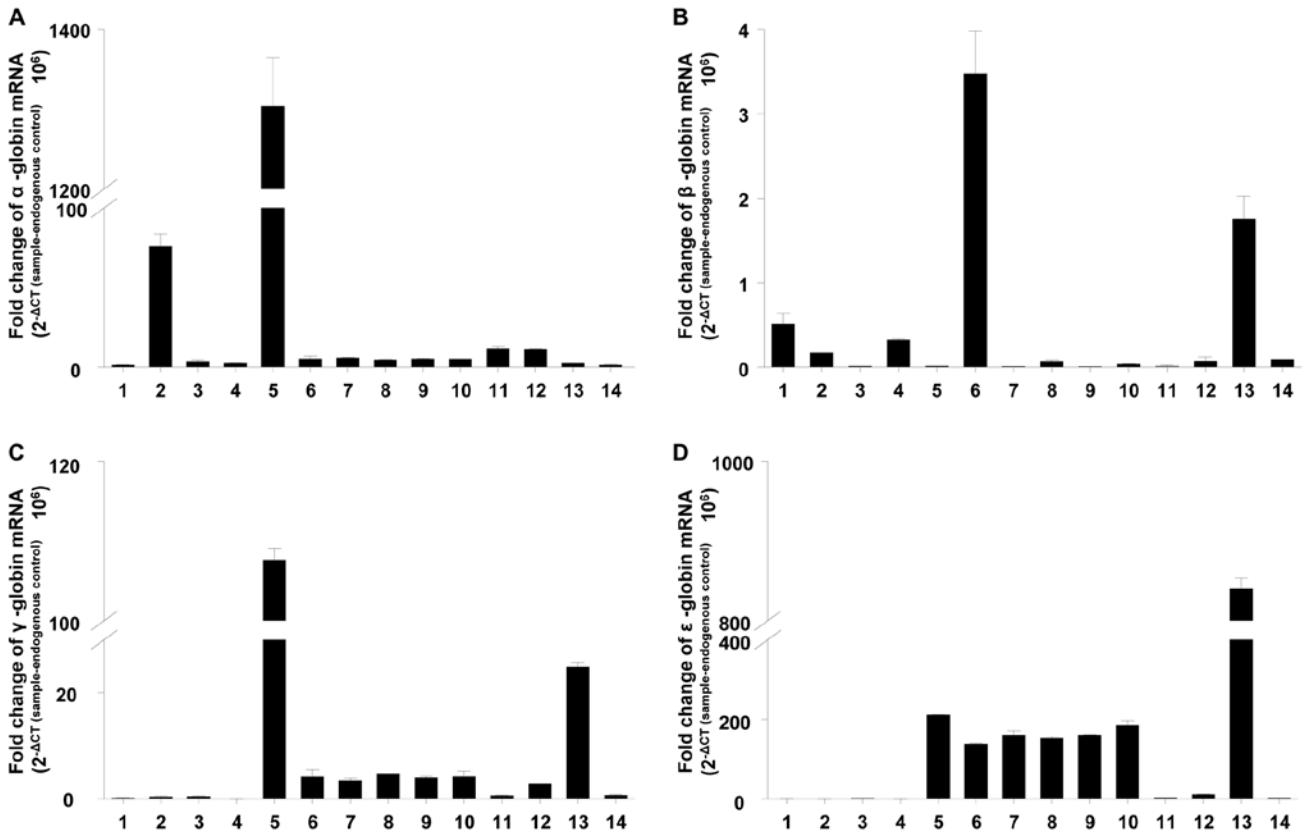

Figure 5. Normalized expression of (A) α-, (B) β-, (C) γ- and (D) ε-globins mRNA in GBM-BTSCs (bars 1, 2, 3, 4 and 5), $CD133^+$ (bar 6) and $CD133^-$ (bar 7) fractions of M006x cells separated by MACS, $CD133^+$ (bar 8) and $CD133^-$ population (bar 9) of M006x cells sorted by flow cytometer and GBM cell lines; M006x (bar 10), M059J (bar 11), M059K (bar 12), U87R (bar 13) and U87T (bar 14). Data are expressed as normalized expression of globin mRNA ($2^{-\Delta CT\ (sample-endogenous\ control)}$ x 10^6) (n=4).

γ globins. A similar pattern of hemoglobin switching occurs in developing erythroblasts during ontogeny when embryonic hemoglobin expression in primitive erythrocytes developing in yolk sac is followed by dominance of the fetal hemoglobin (65). Furthermore, the prevalence of α and γ globin under hypoxia is similar to the predominance of fetal hemoglobin during fetal development that has been related possibly to low O_2 in the fetal hematopoietic microenvironment (66,67).

Hypoxia significantly increases the expression of the cancer stem cell marker CD133 in GBM cell lines (68,69). $CD133^+$ GBM stem cells have been reported to be relatively chemo- and radio-resistant as compared to the bulk GBM cell population (46,47,70,71), and increased pathological grades of astrocytomas have been correlated with increased quantities of CD133 mRNA (72). We therefore compared globin expression profiles on established GBM cell lines and $CD133^+$ and $CD133^-$ GBM cell populations as well as in GBM-BTICs. However, lack of significant differences in expression of different globins in $CD133^+$ and $CD133^-$ fractions of GBM sorted cells, or in GBM-BTICs vs. GBM cell lines, indicates that globin expression is not a stem cell specific characteristic.

Hemoglobin gene switching control has been investigated extensively in an attempt to find a pharmacological approach to hemoglobinpathies such as the thalassemias and sickle cell disease. Most of that effect has been directed at promoting β globin expression so that fetal hemoglobin levels in erythropoietin and their progeny may be increased. Hydroxyurea, thought to work via its S phase inhibitory properties, is useful in ameliorating hemoglobinpathy morbidity in many patients with sickle cell disease and in a limited number of patients with β thalassemia. Another chemotherapy agent, 5-azacytidine, increases fetal hemoglobin production. It was originally thought to work by S phase inhibitors but is now considered to be a DNA methylation inhibitor (73). Another pharmacological approach to the modulation of hemoglobin F production involves histone deacetylase and transferase. An example of how this might be translated to suppression of hemoglobin F production is the p38 signaling involved in MAP kinase activity. Witt *et al* (74) reported that inhibition of the p38 pathway abolished the induction of HbF. This type of small molecule inhibitor treatment may reduce the survival of the BTIC that are exposed to hypoxia.

Although α and β globin expression has been reported in many non-erythroid cells, to our knowledge, we are the first to report the hypoxic upregulation of α, β, γ, δ, ζ and ε globins in human GBM cell lines. Our results also suggest that hypoxic upregulation of globins expression may be to be driven by increased erythropoietin expression, although this has yet to be directly tested. Our results, together with the known non-oxygen transport related functions of hemoglobin, suggest that hemoglobin expression and its hypoxic upregulation in GBM cells may be a part of repertoire of active

defence and adaptation mechanisms by which those cells resist even aggressive multimodality treatments. New therapeutic approaches are required to interfere with hemoglobin expression/or functions in GBM cells.

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