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Expression and hypoxic up-regulation of neuroglobin in human glioblastoma cells

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ABSTRACT

Neuroglobin is a recently identified globin molecule that is expressed predominantly in the vertebrate brain. Neuroglobin expression increases in oxygen-deprived neurons, suggesting it protects neurons from ischemic cell death. We report that neuroglobin transcript and protein are expressed in human glioblastoma cells, and that this expression increases in hypoxia *in vitro*. We also show that neuroglobin is up-regulated in hypoxic microregions of glioblastoma tumor xenografts. Our finding of hypoxic up-regulation of neuroglobin in human glioblastoma cells may provide insight into how tumor cells adapt to and survive in hypoxic microenvironments.

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1. Introduction

Neuroglobin (Ngb), a recently discovered member of the vertebrate globin family, is a small monomeric heme protein with oxygen binding properties (Burmester et al., 2000; Giuffrè et al., 2008). Although Ngb's amino acid sequence shows little similarity to that of hemoglobin or myoglobin, amino acids that confer hemoglobin and myoglobin function are conserved as are all characteristics of the globin fold (reviewed in Brunori and Vallone, 2007). Insertion/deletion events are rare, and approximately half of the positions are strictly conserved (Burmester et al., 2004).

As its name suggests, Ngb is expressed primarily in neuronal tissues, with highest concentrations (~100 μM) found in the retina, localized near mitochondria (Geuens et al., 2003; Pesce et al., 2002). High Ngb levels are also found in the brain

in regions that show greatest oxygen consumption (Hankeln et al., 2004). Lower levels of Ngb are reported in pancreas, adrenal gland and testes, but Ngb is absent from liver, heart, striated muscle, lung, small bowel, kidney and vasculature (Hankeln et al., 2004; Mammen et al., 2002).

Although 8 years have passed since the discovery of Ngb, its physiological function remains elusive (Greenberg et al., 2008). Ngb binds oxygen, however, its concentration in tissues other than retina is too low to suggest it might function as an oxygen delivery molecule (Schmidt-Kastner et al., 2006). Based on its regional (greatest in brain areas with high oxygen consumption) and subcellular (near mitochondria) distributions, Ngb may play an important role in scavenging reactive oxygen or nitrosative species, or by activation of yet unknown protective mechanisms (Brunori and Vallone, 2007; Burmester and Hankeln, 2004; Fordel et al., 2006, 2007b; Greenberg et al.,

Abbreviations: Ngb, neuroglobin; GBM, glioblastoma multiforme; RT, reverse transcription; qRT-PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; DAB, 3,3'-diamino benzidine tetrahydrochloride; GFAP, glial fibrillary acidic protein; HIF-1, hypoxia-inducible factor-1; VEGFR2, vascular endothelial growth factor receptor 2.

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2008; Jin et al., 2008). Studies that have sought evidence for a neuroprotective function *in vivo* have produced conflicting results (Fraser et al., 2006; Hundahl et al., 2005, 2006a; Sun et al., 2001, 2003) that may reflect differences in the duration of hypoxia (acute vs. chronic) or in the methods used to detect Ngf (in situ hybridization vs. immunohistochemistry) (Hankeln et al., 2004). However, *in vitro* studies with neuronal cell lines have shown that hypoxia (<1% O₂) significantly increases Ngf transcript (Fordel et al., 2004; Rayner et al., 2006; Schmidt-Kastner et al., 2006; Sun et al., 2001) and that these increases are associated with protection from hypoxic damage (Greenberg et al., 2008; Sun et al., 2001, 2003).

Glioblastoma multiforme (GBM) is the most common malignant brain tumor in adults. Despite aggressive multimodality therapy, the 2-year survival rate remains only 10–25% (Stupp et al., 2005). The presence of necrosis, and by inference hypoxia, is a pathognomonic feature of human GBM tumors (Wilden and Moore, 1987). Hypoxic tumor cells constitute a source of local treatment failure and disease progression (Dachs and Chaplin, 1998). To survive in hypoxic microenvironments, cells must sense changes in [O₂] and activate defense and adaptation mechanisms (Hochachka et al., 1996). Previous work suggests that up-regulation of Ngf expression may be part of the repertoire of hypoxia defense mechanisms in normal brain. Although most studies have shown that in normal brain, Ngf expression is restricted to neurons, and absent from glia, Chen et al. (2005) reported that Ngf transcript is expressed in cultured astrocytes from newborn mouse brain, and that Ngf protein co-localizes with glial fibrillary acid protein (GFAP) in cultured astrocytes. Further, microarray data publicly available in the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>; accession number E-GEOD-4209) show that Ngf is variably expressed in human GBM tumor samples. Based on these results, we examined whether human GBM cell lines that vary in their ability to survive under hypoxic stress condition also express Ngf, and whether physiologically relevant levels of hypoxia can moderate Ngf expression in these cells.

2. Results

2.1. Ngf transcript and protein are expressed in GBM cell *in vitro*

Ngf mRNA was detected in all GBM cell lines incubated under standard laboratory conditions (controls). When incubated under hypoxic conditions (0.6% O₂), Ngf mRNA was significantly ($P < 0.05$) up-regulated at 24 h (M006x and M006xLo) and 48 h (M010B, M006x, M006xLo, M059K, U87R and U87T) (Figure 1A–G). The single exception was the hypoxia-sensitive cell line, M059J (Figure 1B), in which Ngf mRNA expression was increased ~4-fold, however, this difference was not significant. In accordance with qRT-PCR results, Ngf protein was detected in all GBM cell lines, with variable expression among cells cultured under standard laboratory conditions. Following incubation under hypoxic conditions, Ngf protein was significantly increased at 24 h (M010B and M059K) and 48 h (M010B, M059J, M006x, M006xLo, M059K, U87R and

U87T) (Figure 2A–G). However, there was no correlation between the magnitude of Ngf protein increase after hypoxia and the respective basal levels of each cell line.

2.2. Ngf protein is expressed in GBM xenografts

To test whether Ngf expression is up-regulated under physiologically relevant hypoxic conditions, M006xLo cells were grown as tumor xenografts in NOD/SCID mice. Tumor sections were immunostained with antibodies to Ngf and the hypoxia marker, pimonidazole. Visually distinct regions of necrosis present in serial tumor sections were used as landmarks to compare adjacent tumor sections (Figure 3A). As expected, tumor cells in regions adjacent to necrotic tissue were hypoxic as shown by positive pimonidazole staining (Figure 3B). Strong Ngf immunoreactivity was also detected in the matched tumor regions adjacent to necrosis as well as in scattered foci throughout the tissue section (Figure 3B). Weak Ngf staining was observed throughout the tumor sections, however, these cells lacked pimonidazole immunoreactivity and are thus presumed to have been well oxygenated (Figure 3C).

3. Discussion

The objectives of this study were to determine whether Ngf is expressed in human GBM cells and if so, whether its expression could be modified by physiologically relevant levels of hypoxia. Ngf expression in neuronal tissues is well described, however, there are conflicting reports regarding Ngf expression in non-neuronal brain cells. Double immunostaining of either hippocampal cell cultures or tissue sections of mouse brain and canine retina with antibodies to Ngf and the glial marker GFAP showed no co-localization, suggesting Ngf is absent in cells of glial origin (Laufs et al., 2004; Ostojic et al., 2006). However, using RT-PCR and double immunostaining with Ngf and GFAP antibodies, Chen et al. (2005) reported the presence of Ngf mRNA and protein in cultured astrocytes isolated from mouse brain. Brain sections from a Ngf-overexpressing transgenic mouse showed positive Ngf expression in neuronal cells as well as in cells expressing GFAP (Khan et al., 2007). In the brains of elderly individuals, Ngf has been detected in activated microglial cells and in deep subcortical lesions where it was co-expressed with known hypoxia markers (i.e., HIF1 α , HIF2 α , VEGFR2) (Fernando et al., 2006). Ngf transcript has also been quantified in a microarray analysis of human primary GBM tumor samples (<http://www.ebi.ac.uk/arrayexpress>; accession number E-GEOD-4209). Together, these results indicate that under certain conditions (i.e., hypoxia in normal brain, or metabolically active tumor tissue) Ngf may be expressed in cells that also express glial markers.

Here, we report Ngf mRNA and protein are expressed in seven human GBM cell lines. The cell lines with the “M0xx” designation have been characterized previously for their ability to adapt to and survive under conditions of low oxygen availability, and in contrast to GBM cell lines available from cell repositories, are used at relatively low passage numbers in an attempt to avoid “drift” from the cellular and genetic

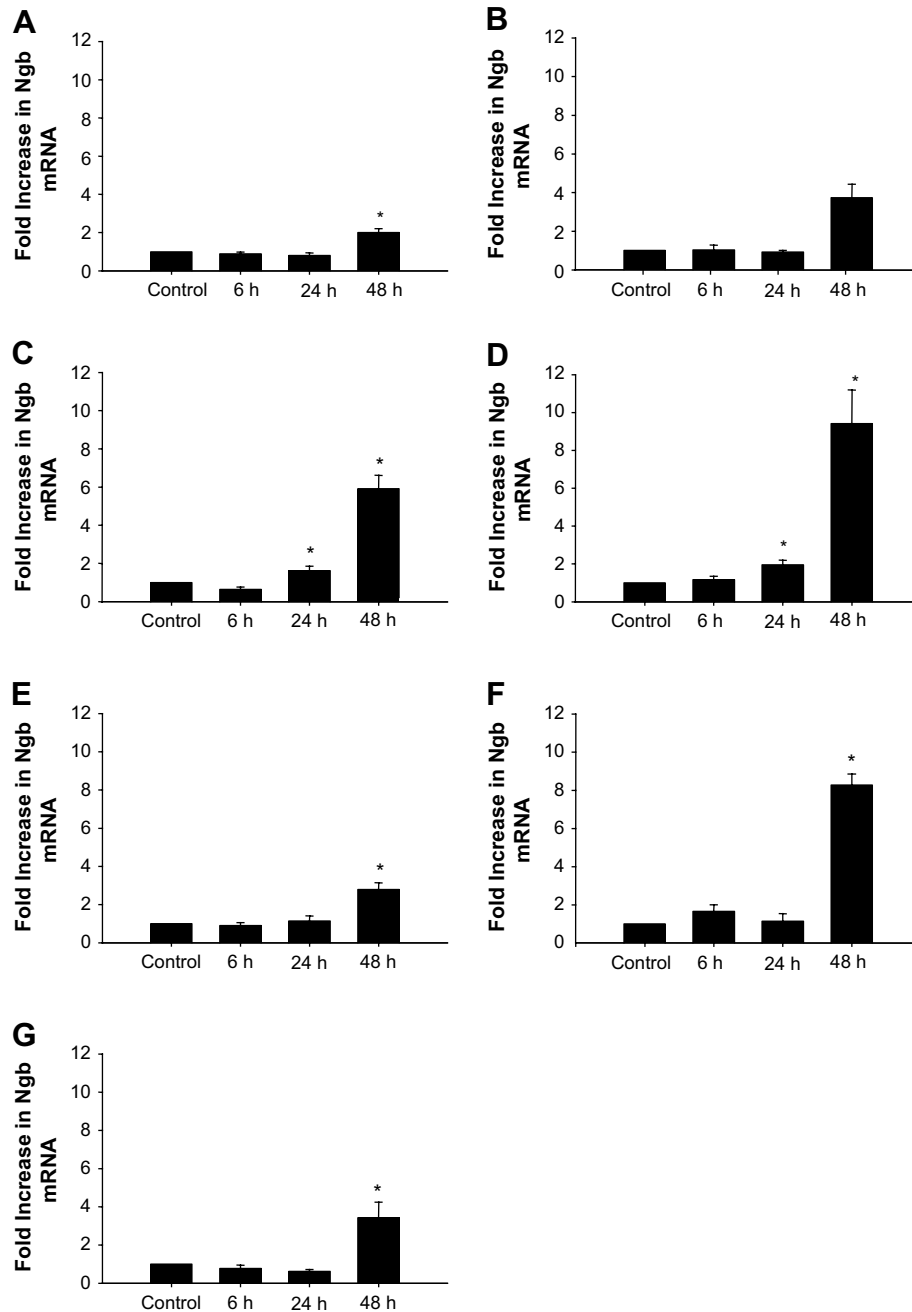


Figure 1 – Effect of hypoxia on Ngb mRNA expression in human GBM cells. (A) M010B; (B) M059J; (C) M006x; (D) M006xLo; (E) M059K; (F) U87R; (G) U87T. Ngb mRNA expression was assessed by qRT-PCR after exposure to hypoxia (0.6% O₂) for 0, 6, 24 and 48 h. Data are expressed as fold increase relative to aerobic control. ($n = 4$). * $P < 0.05$ (ANOVA).

characteristics of the tumor from which they were isolated. The two U87 variant cell lines used here provide examples of GBM cells that differ in their invasiveness properties *in vivo*.

Although GBM are classified as astrocytic tumors, the cellular origin of GBM is not known. GBM cells lines co-express both neural and glial markers (reviewed in Rebetz et al., 2008) and there is evidence that malignant transformation of neuronal stem cells may lead to the development of GBM tumors (Sanai et al., 2005; Singh et al., 2004a,b), but this remains controversial (Stiles and Rowitch, 2008). Thus, it is not entirely

unexpected that Ngb, a protein originally identified in neurons, may be present in GBM cells.

The cellular response to hypoxia has been characterized in five of the cell lines: M006x, M006xLo, M059K are hypoxia-tolerant whereas M010B and M059J are hypoxia-sensitive (Allalunis-Turner et al., 1999; Parliament et al., 2000; Turcotte et al., 2002). M006xLo cells are of particular interest as they were selected from the M006x parental line following culture for 2 weeks under reduced oxygen and glucose conditions, and accordingly, are exceptionally tolerant of low oxygen

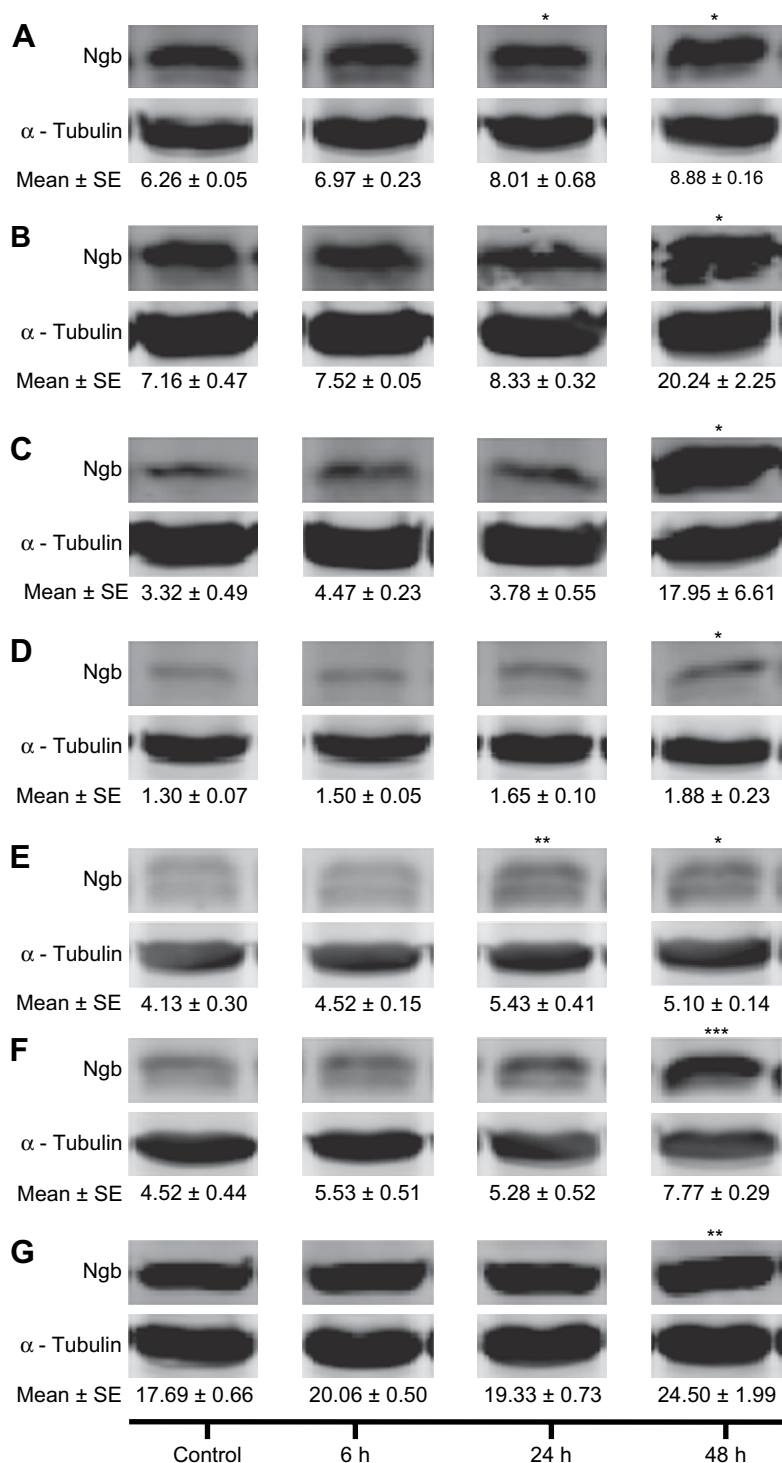


Figure 2 – NgB protein expression in human GBM cells. (A) M010B; (B) M059J; (C) M006x; (D) M006xLo; (E) M059K; (F) U87R; (G) U87T. NgB expression was assessed by Western blot analyses after exposure to hypoxia (0.6% O₂) for 0, 6, 24 and 48 h. The integrated intensities of NgB and α-tubulin (control) bands were determined and expressed in arbitrary units (AU), and representative blots are shown. (n = 4) *P < 0.05; **P < 0.01; ***P < 0.001 (ANOVA).

conditions. Both NgB transcript and protein were detected in all GBM cell lines tested, although basal levels of expression varied. The M006xLo cell line was shown overall to have the lowest protein expression, and the two hypoxia-sensitive cell lines, the highest. If NgB functions to protect cells from

hypoxia/oxidative stress, then these results may seem counterintuitive. However, the repertoire of cellular defense mechanisms involves multiple pathways, and tumor cells that have acquired the ability to survive under low oxygen conditions have likely up-regulated a variety of adaptive mechanisms.

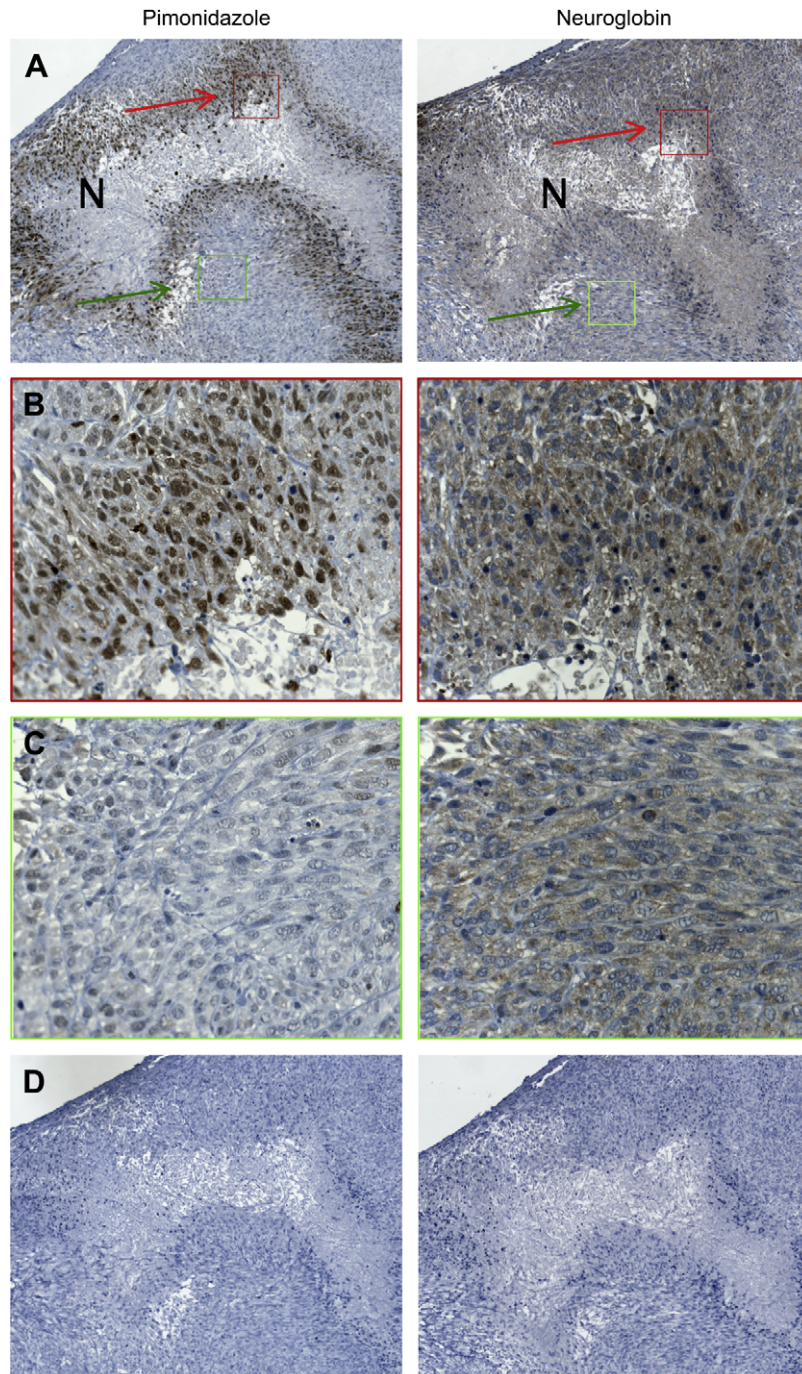


Figure 3 – NgB expression in a M006xLo tumor xenograft. (A) Low-power (10 \times) view of adjacent serial sections stained with antibodies to pimonidazole or NgB. Regions of necrosis (N) were used as tissue landmarks. **(B)** High-power views (40 \times) of regions indicated by the red boxes in panel A. Hypoxic cells identified by pimonidazole staining also show strong positive staining for NgB. **(C)** High-power views (40 \times) of regions indicated by the green boxes in panel A. Weakly positive NgB staining was also observed throughout the tumor section, however, these cells showed no pimonidazole immunoreactivity. **(D)** Low power view (10 \times) of control tumor sections from which primary antibodies were omitted.

Thus, while NgB may function as a protective molecule during hypoxia or ischemia/reperfusion, and may show robust expression in hypoxia-sensitive cell types such as normal neurons and M010B and M059J tumor cells, its expression may be less critical in cells that have constitutively up-regulated adequate hypoxia defense mechanisms.

Both NgB transcript and protein were significantly increased in GBM cells cultured under hypoxic conditions. For these experiments, cells were incubated in atmospheres of 0.6% O₂, a concentration of oxygen that is within the range of concentrations that have been measured in the hypoxic regions of human tumors (Olive et al., 1996; Vaupel et al., 1991).

These results are similar to those previously reported for neuronal cell lines and cultures in which increases in Ngb mRNA were observed following exposure to hypoxia (Schmidt-Kastner et al., 2006; Sun et al., 2001) or the hypoxia-mimetic agent, cobalt chloride (Sun et al., 2001). However, the 2- to 9-fold increase in Ngb mRNA levels observed in GBM cells after 48 h of hypoxia is higher than previously reported for neuronal cells (Fordel et al., 2004; Rayner et al., 2006; Schmidt-Kastner et al., 2006; Sun et al., 2001). The use of different cell types and experimental conditions to induce hypoxia may explain differences in the magnitude of the Ngb response. In our work and the studies noted above, no attempt was made to control for differences in glucose consumption rate or changes in pH that might have existed between control and hypoxic cultures. Fordel et al. (2007a) have reported that Ngb mRNA and protein are up-regulated in a human neuroblastoma cell line after combined oxygen–glucose deprivation, but not after hypoxia (1% O₂) alone or glucose deprivation alone.

Among the cell lines in this study, there was no obvious relationship between basal Ngb protein levels and the extent to which its expression was up-regulated by hypoxia, nor was there a direct relationship between hypoxia-induced increases in Ngb transcript and protein. Analysis of relationships between transcript and protein expression in human lung cancer and hematopoietic stem cells has shown that only a subset of mRNAs show significant positive correlation between transcript–protein expression levels (Chen et al., 2002; Unwin et al., 2006). The transcriptional and/or translational control of Ngb expression is incompletely understood, as are factors that regulate Ngb protein stability or turnover. Using *in silico* techniques, Wystub et al. (2004) showed, somewhat unexpectedly, that conserved hypoxia-response elements, including HIF-1 consensus binding motifs, are absent from the upstream region of human NGB. However, the NGB promoter region does contain AP-1 and NFκB binding motifs, both of which have been reported to be activated by hypoxia.

In addition to differences in hypoxia sensitivity, there are other notable phenotypic differences among these GBM cells. The intrinsic radiosensitivity of the M0xx-designated cells varies widely (Allalunis-Turner et al., 1992). Also of note, the M059J is the only example of a human tumor cell line that lacks expression of DNA-PK_{cs}, a critical component of the non-homologous end-joining pathway responsible for repair of DNA double strand breaks (Lees-Miller et al., 1995). The absence of DNA-PK_{cs} renders M059J cells exquisitely radiosensitive (Allalunis-Turner et al., 1993). Although the rank order of cellular radiosensitivities does parallel the differences in basal Ngb protein expression, at present there is no evidence for a direct role for Ngb in the repair of DNA double strand breaks, the mechanism believed to underlie differences in cellular radiosensitivity.

To test whether physiologically relevant hypoxic conditions could increase Ngb expression, we examined the distribution of Ngb in tissue sections of a GBM xenograft. Pimonidazole is a bioreductive hypoxia marker that irreversibly binds to hypoxic cells at oxygen tensions <10 mm Hg (Yaromina et al., 2006), and is a well accepted marker of tumor hypoxia in tumor specimens (Ljungkvist et al., 2007). Interestingly, we observed enhanced pimonidazole and Ngb staining

in tumor regions adjacent to necrosis. The presence of hypoxic cells adjacent to necrotic tissue conforms to the classic model of tumor hypoxia (Brown and Wilson, 2004). Thus, it is reasonable to postulate that Ngb expression *in vivo* also may be controlled by oxygen-dependent regulatory mechanisms.

Previous studies that examined Ngb expression *in vivo* reported increases in Ngb protein in brain neuronal cells after hypoxia or ischemic injury (Fordel et al., 2004, 2007b; Hundahl et al., 2006b; Li et al., 2006; Rayner et al., 2006; Schmidt-Kastner et al., 2006; Sun et al., 2001), suggesting it functions as an endogenous neuroprotectant (Greenberg et al., 2008; Sun et al., 2001, 2003). Consistent with this, Chen et al. (2005) observed that transfection of cultured astrocytes with anti-sense Ngb oligonucleotide led to a 2.5-fold increase in apoptotic cells after 5 h of ischemic treatment. The report of Ngb overexpression in the brain of an individual with hereditary ferritinopathy also is consistent with Ngb's putative function as a radical scavenger (Powers, 2006). Hereditary ferritinopathy is a rare neuro-genetic disorder caused by mutations in the *L-ferritin* gene, resulting in an accumulation of iron that leads to severe oxidative stress. Peroxynitrite and products of lipid peroxidation are believed to play a pathogenic role in this disorder (Mancuso et al., 2005). In this patient, Ngb overexpression was localized to swollen neuronal and glial nuclei, the same cells that also showed over expression of ferritin and iron accumulation. Powers (2006) suggested that Ngb expression may be an initial protective response against iron-initiated oxidative damage. However, if the protective response is ineffective or overwhelmed, as would be the case in hereditary ferritinopathy, then Ngb release from dead cells could accelerate iron stress as Ngb heme is converted to iron by heme-oxygenase. A possible association between ferritin and Ngb expression in human GBM has yet to be explored. Ferritin is an iron binding protein that protects cells against iron-catalyzed oxidative stress, and elevated ferritin levels have been reported in the cerebral spinal fluid of patients with GBM (Sato et al., 1998). In human GBM tumor sections, elevated ferritin expression has also been observed in tumor cells within the invasive edge and in microglia, but ferritin was absent from neurons and reactive astrocytes (Holtkamp et al., 2005). Additional studies will be required to determine whether ferritin and Ngb are co-expressed in human GBM and if these proteins function in tandem to increase tumor cell resistance to oxidative stress.

Mechanisms that allow tumor cells to adapt to and survive in hypoxic microenvironments include both metabolic adaptation and transcriptional activation of hypoxia rescue genes. Our findings that Ngb expression increases in GBM cells in response to hypoxia, both *in vitro* and *in vivo*, suggest Ngb may be part of the repertoire of defense strategies that GBM cells utilize to survive in hypoxic microenvironments. Human tumors frequently contain hypoxic cells and their presence is a poor prognostic indicator (Brown and Wilson, 2004). For example, compared to well oxygenated tumor cells, hypoxic tumor cells are approximately 3-fold resistant to ionizing radiation and certain chemotherapeutic agents, are genetically unstable and metastasize frequently (Dachs and Chaplin, 1998). With regard to treatment outcome, patients whose tumors contain a significant proportion of hypoxic cells have worse local-regional control and progression-free survival following surgery

alone or surgery plus radiotherapy (Hockel et al., 1993, 1998; Nordmark and Overgaard, 2000). There is also evidence that hypoxia *per se* selects for a more aggressive tumor phenotype (Bristow and Hill, 2008). The identification of Ngb expression in human glioblastoma cells, and its up-regulation by hypoxia, provides additional insight into the pro-survival mechanisms utilized by these aggressive cancers. Strategies to interfere with Ngb expression and/or function may ultimately result in therapeutic benefit.

4. Experimental procedures

4.1. Cell lines and in vitro culture conditions

The origin and characterization of the following GBM cell lines have been published previously: M059J (ATCC number CRL2366), M010b – hypoxia-sensitive; M059K (ATCC number CRL2365), M006x, M006xLo – hypoxia-tolerant (Allalunis-Turner et al., 1992, 1999; Parliament et al., 2000). The U87T and U87R GBM cell lines were established following serial *in vivo* selection in a glioma mouse model (Johnston et al., 2007). U87T cells were isolated from the actively growing tumor mass of an intra-cerebral U87 tumor; invasive U87R cells were isolated from the contra-lateral brain. Both cell lines were kindly provided by Dr Donna Senger (University of Calgary). Cells were maintained as monolayer cultures in DMEM/F12 media supplemented with 10% fetal calf serum, 1 mM L-glutamine, and 100 U penicillin G/ml in a humidified atmosphere of 5% CO₂ in air at 37 °C. All tissue culture supplies were purchased from GIBCO.

4.2. Hypoxia labeling of tumor xenografts

Animal experiments were performed according to guidelines for the use of animals in research established by the Canadian Council for Animal Care and were approved by the Animal Care Committee at the Cross Cancer Institute. M006xLo tumor xenografts were initiated in NOD/SCID mice by intra-dermal injection of 10⁶ to 10⁷ cells in both flanks. When the tumor volumes equaled 100–300 mm³, mice received a single injection of the hypoxia marker, pimonidazole HCl (100 mg/kg *i.p.*, Natural Pharmacia International). Ninety minutes later, mice were euthanized, and the tumors excised, fixed in buffered formalin, embedded in paraffin and then sectioned at 5 µm intervals.

4.3. Generation of hypoxia in vitro

A de-gassing manifold designed by Dr Cameron Koch was used to generate hypoxia *in vitro* (Koch et al., 1979). Exponential phase cells (~2 × 10⁵) were seeded onto 60-mm glass plates and then incubated under standard laboratory culture conditions (5% CO₂ 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% CO₂/balance N₂ until an oxygen tension of 0.6% was achieved. The sealed, air-tight aluminum chambers were then incubated at 37 °C for 6–48 h. At the end of each incubation interval, the aluminum chambers were unsealed,

the tissue culture plates removed and RNA and protein isolated from the cells as described in Section 4.4.

4.4. Quantitative real-time reverse transcription-PCR

Total RNA from cultured cell lines was isolated using the RNeasy mini kit (QIAGEN). Reverse transcription (RT) was carried out with 1 µg total RNA per 20 µl reaction volume using QuantiTect reverse transcription kit (QIAGEN). RT experiments were performed with GeneAmp PCR system 9700 (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) analysis was performed with a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan fast universal PCR master mix and a validated TaqMan Gene Expression assay (Applied Biosystems) for the human NGB gene (assay ID: Hs00222034_ml). Human 18S rRNA gene was used as endogenous control. Amplification data were analyzed with SDS RQ Manager 1.2. Relative quantities of Ngb transcripts were normalized against relative quantities of the 18S rRNA transcripts, and fold-expression changes calculated using the equation $2^{-\Delta\Delta CT}$.

4.5. Western blotting

Whole-cell lysates were prepared using complete Lysis-M buffer (Roche Diagnostics) and protein content determined using a protein assay kit (Pierce). Equal amounts of protein (50 µg) were resolved using 13% SDS-PAGE under reducing condition and electro-transferred to nitrocellulose membranes. Membranes were blocked with 5% donkey serum then incubated with Ngb antibody (1:1000 rabbit anti-human polyclonal antibody, Sigma) or α -tubulin antibody (1:5000, mouse anti-human α -tubulin monoclonal antibody, Sigma) as loading control. Membranes were incubated with IRDye[®] 800 donkey anti-rabbit (1:5000, Rockland Immunochemicals for Research) or Alexa Fluor[®] 680 donkey anti-mouse (1:5000, Molecular Probes, Invitrogen) and scanned using the Odyssey Infrared Imaging System (Li-COR Biosciences). Bands were analyzed using Odyssey application software version 2.1 and the integrated areas of bands were determined and expressed in arbitrary units (AU).

4.6. Tumor tissue immunostaining

Detection of pimonidazole adducts was as previously described (Kleiter et al., 2006) with modifications. Tissue endogenous peroxidase was quenched by incubation with 3% H₂O₂ for 15 min. Sections were processed for antigen retrieval, incubated with blocking buffer (0.5 M glycine in PBS-0.2% brij35) and then with antibodies to Ngb (1:150, mouse anti-human Ngb monoclonal antibody, BioVendor Laboratory Medicine Inc., Czech Republic) or pimonidazole (1:500, rabbit anti-pimonidazole polyclonal antibody, Natural Pharmacia International). Tissue sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse antibodies (DakoCytomation) and positive staining visualized by the chromogenic reaction of HRP with DAB (DakoCytomation).

4.7. Statistical analyses

Data from four replicate experiments were expressed as mean ± standard error. Statistical analyses were performed

using SigmaPlot 11 software (Systat Software, Inc). 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, all pairwise multiple comparison procedures were carried out using Fisher Least Significant Difference, Dunn's method or Student–Newman–Keuls method as post-hoc tests.

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