Aus dem Fachbereich Medizin
der Johann Wolfgang Goethe-Universität
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betreut am
Zentrum der Neurologie und Neurochirurgie
Klinik für Neurologie -
Funktionsbereich Neuroonkologie
Leitung: Prof. Dr. Joachim P. Steinbach

Die Bedeutung des Serin-Metabolismus für die Redox-Homöostase und das Überleben von Glioblastomzellen im Tumormikromilieu

Dissertation
zur Erlangung des Doktorgrades der Medizin
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vorgelegt von
Anna Larissa Engel

aus Wiesbaden

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Zusammenfassung

Das Glioblastom ist der häufigste bösartige primäre Hirntumor im Erwachsenenalter. Trotz intensiver Forschungsbemühungen inklusive zahlreicher Therapiestudien liegt die mediane Gesamtüberlebenszeit selbst von Patienten in gutem Allgemeinzustand noch immer unter zwei Jahren. Deshalb ist die Erforschung möglicher Resistenzmechanismen sowie neuer therapeutischer Angriffspunkte dringend erforderlich, um effizientere Therapien zu entwickeln.

Insbesondere der Tumormetabolismus hat in den vergangenen Jahren an Bedeutung in der Krebsforschung gewonnen. Hier zeigte sich in anderen Tumorentitäten, dass der Metabolismus der Aminosäure Serin, insbesondere des Schlüsselenzyms der Serin-Biosynthese, der 3-Phosphoglyceratdehydrogenase (PHGDH), ein Ansatzpunkt für neue Therapien sein kann. Wir stellten uns die Frage, ob auch im Glioblastom der Serinstoffwechsel von Bedeutung für Wachstum und Überleben der Tumorzellen ist und ob dieser somit einen neuen therapeutischen Angriffspunkt darstellen kann.

Zur Untersuchung dieser Fragestellung wurden initial verschiedene Tumorzelllinien hinsichtlich ihrer basalen Expression von PHGDH und weiterer Schlüsselenzyme des Serinstoffwechsels auf mRNA- und Protein-Ebene analysiert. Anschließend wurde untersucht, ob sich die Expression dieser Enzyme durch Zellstress verändert. Zur weiteren Evaluierung der PHGDH als möglichem Ansatz für neue Therapien wurde deren Aktivität pharmakologisch und genetisch inhibiert. In Wachstumsanalysen wurde ferner die Abhängigkeit der Zellen von verschiedenen Serinquellen untersucht. Unter Bedingungen des Tumormikromilieus wurden Zelltod- und Redox-Stress-Parameter untersucht. Abschließend wurden die Ergebnisse durch eine PHGDH-Überexpression validiert.

Die basalen Expressionsniveaus der PHGDH unterschieden sich in den fünf analysierten Gliomzelllinien. Es zeigte sich keine Korrelation mit der Expression der Serinhydroxymethyltransferase 1 und 2 (SHMT 1 und 2, zytoplasmatische bzw. mitochondriale Isoform), welche ebenfalls Schlüsselenzyme im Serinstoffwechsel

sind. Unter Zellstress, welcher mittels Hypoxie oder Aktivierung des Nuclear factor erythroid 2-related factor 2 (Nrf2) induziert wurde, zeigte sich ein Anstieg der Expression von PHGDH und SHMT2. Im nächsten Schritt erfolgten Versuche mit CBR-5884, einem pharmakologischen Inhibitor der PHGDH. CBR-5884 reduzierte signifikant die intrazellulären Serin- und Glycin-Spiegel. Parallel zeigte sich eine dosisabhängige, signifikante Reduktion des Zellwachstums in allen getesteten Linien, ohne eine Induktion von Zelltod. Zelllinien mit niedriger PHGDH-Expression wurden zusätzlich bereits durch Entzug von Serin aus dem Kulturmedium signifikant in ihrem Wachstum gehemmt. Unter realistischen Bedingungen des Tumormikromilieus mit Sauerstoff- und Nährstoffmangel zeigte sich unter zusätzlicher PHGDH-Inhibition mittels CBR-5884 ein signifikanter Anstieg von Zelltod-Parametern und reaktiven Sauerstoffspezies (ROS) einhergehend mit einer reduzierten $NADPH/NADP^{+}$-Ratio. Die Ergebnisse der pharmakologischen PHGDH-Inhibition konnten durch eine PHGDH-Gensuppression reproduziert werden. Umgekehrt bestätigte eine genetische PHGDH-Überexpression den protektiven Effekt eines aktiven Serinmetabolismus für die Tumorzellen.

Zusammenfassend zeigte sich in unseren Versuchen, dass unter Bedingungen des Tumormikromilieus einige Gliomzell-Linien den Serinmetabolismus induzieren, was einen protektiven Effekt für das Überleben unter widrigen Mikromilieusbedingungen zu haben scheint. Umgekehrt reduzierte eine Inhibition der PHGDH als Schlüsselenzym der Serinsynthese unter diesen Bedingungen das Wachstum von Glioblastom-Zellen und induzierte gleichzeitig Zelltod und Redoxstress. Diese Ergebnisse rechtfertigen eine weitere präklinische Testung in *in vivo* Modellen. Zusammenfassend legen unsere Ergebnisse nahe, dass der Serinmetabolismus einen vielversprechenden Angriffspunkt für neue Therapiestrategien im Glioblastom darstellen könnte.

Summary

Glioblastoma is the most common malignant primary brain tumour in adulthood. Despite intensive research efforts including numerous therapy studies, the median overall survival of patients even in good clinical conditions remains below two years. Therefore, research into possible resistance mechanisms as well as new therapeutic targets are urgently needed in order to develop more efficient therapeutic approaches.

Tumour metabolism has achieved increasing relevance in cancer research within the last years. In other tumour entities, it has been shown that the metabolism of the amino acid serine, in particular of the key enzyme of serine biosynthesis, 3-phosphoglycerate dehydrogenase (PHGDH), may represent a new therapeutic target. We asked ourselves whether serine metabolism is also of importance for growth and survival of glioblastoma cells and whether it can thus represent a new therapeutic target.

To investigate this question, we initially analysed different tumour cell lines regarding their basal expression of PHGDH and other key enzymes of the serine metabolism on mRNA and protein level. Subsequently, it was investigated whether the expression of these enzymes is altered by cell stress. For further evaluation of PHGDH as a possible therapeutic target, its activity was pharmacologically and genetically inhibited. In growth analyses, the dependency of the cells on various serine sources was investigated. Cell death and redox stress parameters were investigated under conditions of the tumour microenvironment. Finally, the results were validated by PHGDH overexpression.

The basal expression levels of PHGDH differed in the five analysed glioma cell lines. No correlation was found with the expression of serine hydroxymethyltransferases 1 and 2 (SHMT 1 and 2, cytoplasmic and mitochondrial isoform), which are also key enzymes of serine metabolism. Under cell stress induced by hypoxia or activation of the nuclear factor erythroid 2-related factor 2 (Nrf2), the expression of PHGDH and SHMT2 was increased. In the next step, experiments were performed with CBR-

5884, a pharmacological inhibitor of PHGDH. CBR-5884 significantly reduced intracellular serine and glycine levels. In parallel, a dose-dependent significant reduction of cell growth was shown in all tested lines without induction of cell death under these conditions. In addition, cell lines with low PHGDH levels were significantly inhibited in their growth by the removal of serine from the culture medium. Under realistic conditions of the tumour microenvironment with oxygen and nutrient deficiency, a significant increase in cell death and reactive oxygen species (ROS) was observed with a reduced NADPH/$NADP^+$ ratio under additional PHGDH inhibition by CBR-5884. The results of pharmacological PHGDH inhibition could be reproduced by PHGDH gene suppression. Conversely, genetic PHGDH overexpression confirmed the protective effect of an active serine metabolism on tumour cells.

In conclusion, our experiments showed that under conditions of the tumour microenvironment some tumour cells induce serine metabolism, which thus exerts a protective effect on survival under adverse microenvironmental conditions. Conversely, inhibition of PHGDH, a key enzyme in serine synthesis, reduced growth of glioblastoma cells and simultaneously induced cell death and redox stress under these conditions. These results justify testing in *in vivo* models. In summary, our results indicate that serine metabolism represents a promising target for new therapeutic strategies to improve treatment for glioblastoma.

Abkürzungsverzeichnis

FACS .... Fluorescence Activated Cell Sorting
FCS .... Fetales Kälberserum
LDH .... Laktatdehydrogenase
NADPH .... Nicotinamidadenindinukleotidphosphat
Nrf2 .... Nuclear factor erythroid 2-related factor 2
PHGDH 3-Phosphoglyceratdehydrogenase
PI .... Propidiumiodid
qPCR .... quantitative Polymerasekettenreaktion
ROS .... Reaktive Sauerstoffspezies
SHMT1 .... Serinhydroxymethyltransferase 1
SHMT2 .... Serinhydroxymethyltransferase 2
WHO .... World Health Organization

Übergreifende Zusammenfassung

Einleitung

Das Glioblastom ist der häufigste maligne hirneigene Tumor beim Erwachsenen[1]. Trotz intensiver Forschungsbemühungen ist die Prognose von Patienten mit Glioblastom weiterhin schlecht. Die aktuelle Erstlinientherapie umfasst neben der primären Resektion eine kombinierte Radiochemotherapie. Kürzlich konnte gezeigt werden, dass die Hinzunahme von Tumortherapiefeldern zu dieser Primärtherapie das mediane Gesamtüberleben in der Studienpopulation auf nun insgesamt 21 Monate verlängert.[2,3] In der Rezidivsituation gibt es jedoch bislang keinen etablierten Therapiestandard. Deshalb ist es unverzichtbar, weiterhin Grundlagenforschung zu betreiben, um potenzielle neue Therapieansätze zu entwickeln.

Das Glioblastom zeichnet sich in seinem Tumormikromilieu durch Regionen mit ausgeprägtem Nähr- und Sauerstoff-Mangel (Hypoxie) aus[4-6]. Unter diesen hypoxischen Bedingungen sowie durch Chemotherapie kann es zu einer Störung der zellulären Redox-Homöostase mit einem konsekutiven Anstieg der reaktiven Sauerstoffspezies (ROS) kommen[7-9].

Seit einigen Jahren ist die zentrale Rolle des Tumormetabolismus für den neoplastischen Phänotyp zunehmend ins Interesse der Krebsforschung gerückt[10]. Aufbauend auf die Beschreibung des Warburg-Effekts der aeroben Glykolyse wurden dabei viele Mechanismen der differenziellen metabolischen Aktivität von Tumorzellen im Vergleich zu nicht transformierten Zellen weitreichend aufgeklärt[11].

In verschiedenen Tumorentitäten wie Lungen-, Brust- und Hautkrebs zeigen aktuelle Arbeiten eine Induktion des Serinmetabolismus bei aggressiveren Tumor-Subtypen[12-14]. Zudem erwies sich bei limitierter Serinverfügbarkeit die endogene Serin-Biosynthese per PHGDH als relevanter Faktor für das Tumorzellwachstum[15]. Parallel dazu wurden neue *small-molecule* Inhibitoren für dieses Schlüsselenzym des Serinmetabolismus entwickelt[14].

Prinzipiell kann Serin als nicht-essenzielle Aminosäure über mehrere Aminosäuretransporter von extrazellulär importiert werden[16,17], aber auch von den Zellen selbst aus 3-Phosphoglycerat, einem Stoffwechselprodukt der Glykolyse, per PHGDH synthetisiert werden[18,19]. Alternativ kann Serin auch aus Glycin gebildet werden, hierbei spielen die zytosolische Serinhydroxymethyltransferase 1 (SHMT1) sowie die mitochondrial vorliegende SHMT2 wichtige Rollen[20]. Im Rahmen der weiteren Verstoffwechselung von Serin kommt es unter anderem auch zur Produktion von NADPH, welches zur Regeneration von Glutathion und damit der Aufrechterhaltung der zellulären Redox-Homöostase benötigt wird[21,22].

In Gliomen ist eine vermehrte PHGDH-Expression mit zunehmendem WHO-Grad sowie vermindertes Zellwachstum und verminderte Invasion bei PHGDH-*Silencing* beschrieben worden[23]. Zudem wurde in perinekrotischen Zonen von Glioblastomen eine vermehrte Expression von SHMT2 beobachtet[24].

Fragestellung

Wir stellten uns die Frage, ob ein veränderter Serinmetabolismus im Glioblastom einen Adaptationsmechanismus an Bedingungen des Tumormikromilieus darstellt und inwiefern dies als therapeutischer Angriffspunkt genutzt werden kann.

Methodischer Ansatz

Zur Untersuchung der Fragestellung untersuchten wir zunächst ein Panel von Tumorzelllinien hinsichtlich ihrer basalen PHGDH-Expression mittels qPCR. Die Proteinexpression wurde anschließend mittels Western Blot evaluiert. Bei einer Auswahl der getesteten Linien untersuchten wir zusätzlich die mRNA- und Protein-Expression der Isoformen der SHMT. Zur weiteren Charakterisierung etwaiger Anpassungsmechanismen an das Tumormikromilieu verwendeten wir Inkubationsschränke mit 1% Sauerstoff, GasPak-Beutel zur Induktion von 0,1% Sauerstoff sowie spezielle Kulturmedien. Hierbei konnten wir durch

Supplementierung von fetalem Kälberserum (FCS), Glucose sowie Serin verschiedene Bedingungen simulieren.

Zur pharmakologischen Inhibition der Serin-Biosynthese verwendeten wir CBR-5884, einen neuen *small-molecule* Inhibitor der PHGDH. Die Bestimmung intrazellulärer Aminosäurekonzentrationen wurde durch Massenspektroskopie durchgeführt. Wachstumsanalysen wurden durch Färbung mit Kristallviolett und photometrischer Auswertung durchgeführt. Zur Untersuchung der Zellviabilität bestimmten wir ebenfalls photometrisch die Laktatdehydrogenase (LDH)-Freisetzung. Reaktive Sauerstoff-Spezies wurden per FACS-Analyse und die NADPH/NADP+-Ratio ebenfalls lumineszenzbasiert gemessen.

Die genetischen Veränderungen wurden zur PHGDH-Gensuppression per Einbringung eines pLKO.1-Plasmids mit entsprechender Zielsequenz durchgeführt. Die induzierbare Überexpression der PHGDH erfolgte durch Einbringen eines pTetOne-Vektors und die Induktion jeweils durch Zugabe von Doxycyclin.

Ergebnisse

Die basale Expression der PHGDH als Schlüsselenzym der Serin-Biosynthese zeigte in den getesteten Zelllinien große Schwankungen. Es zeigten sich sowohl Zelllinien mit sehr niedriger PHGDH-Expression wie LN-308 als auch solche mit sehr hoher wie G55. Als Referenz verwendeten wir MDA-MB-231 und -464, zwei Brustkrebslinien mit beschriebener sehr niedriger beziehungsweise sehr hoher PHGDH-Expression[13]. Wir testeten ebenfalls die Expression der SHMT1 und 2.

Interessanterweise zeigte sich unter hypoxischen Bedingungen eine Steigerung der Expression von PHGDH und SHMT2 in drei der fünf getesteten Zelllinien. Die SHMT1 hingegen wurde nicht induziert. Analoge Ergebnisse zeigten sich auch unter Verwendung eines Nrf2-Aktivators als Modell für Zellstress. Auch hier wurde die Expression von PHGDH und SHMT2, nicht aber von SHMT1 induziert.

Zur weiteren Untersuchung verwendeten wir den neuen *small molecule* Inhibitor der PHGDH, CBR-5884. Wir konnten zeigen, dass dieser die intrazellulären Konzentrationen von Serin und Glycin, nicht aber von anderen Aminosäuren wie Asparagin und Tyrosin, senkt.

Zur Wachstumsanalyse wurde der Inhibitor in zwei Konzentrationen, 30 µM entsprechend der publizierten IC_{50}, und 60 µM verwendet. Unter serumhaltigen Standard-Kulturbedingungen zeigte sich nach einer Woche ein dosisabhängiger Effekt mit signifikanter Wachstumsreduktion in allen getesteten Zelllinien. Auch unter serumfreien Bedingungen wurde das Wachstum aller Zelllinien stark reduziert. Auffällig war hierbei, dass auch LN-308 als die Zelllinie mit niedrigster basaler PHGDH-Expression auf den PHGDH-Inhibitor ansprach. Gleichzeitig zeigte sich hier aber auch schon ein signifikant vermindertes Wachstum durch Entzug von Serin aus dem Kulturmedium. Bei Serumentzug zeigte sich auch bei LNT-229 als Zelllinie mit mittlerem PHGDH-Expressionsniveau durch Serinentzug ein vermindertes Tumorzellwachstum.

Im nächsten Schritt untersuchten wir die Zelllinien unter Bedingungen des Tumormikromilieus. Hierfür verwendeten wir glucosefreies Medium, das mit 2 mM Glucose supplementiert wurde, und induzierten Hypoxie mit Sauerstoff-Konzentrationen von nur 0,1% O_2. Hierbei zeigte sich in allen getesteten Zelllinien ein signifikanter Anstieg der LDH-Freisetzung als Zelltodmarker unter Behandlung mit dem PHGDH-Inhibitor CBR-5884. In durchgeführten PI-FACS-Analysen bestätigten sich diese Ergebnisse. Erneut war in LN-308 und LNT-229 unter den herrschenden Mangelbedingungen allein durch Serin-Entzug aus dem Medium ein signifikanter Effekt auf die LDH-Freisetzung zu beobachten.

Parallel zeigte sich auch eine Erhöhung der freien Sauerstoffradikale. Einhergehend damit ergab sich eine Erniedrigung des intrazellulären Verhältnisses von NADPH zu $NADP^+$.

Um zu klären, ob die beobachteten Effekte PHGDH-spezifisch waren und nicht durch *off-target* Effekte erklärt sein können, stellten wir genetisch veränderte

Zelllinien her. Mit Zellen mit einer stabilen Gensuppression von PHGDH wiederholten wir die Experimente zur Validierung der beobachteten Effekte auf Wachstum und Zelltod. Die Knockdown-Zellen zeigten analog zu den Zelllinien mit einer intrinsisch niedrigen Expression von PHGDH bei Serin-Entzug aus dem Kulturmedium ein signifikant vermindertes Zellwachstum. Auch zeigte sich unter Bedingungen des Tumormikromilieus eine Sensibilisierung für Hypoxie-induzierten Zelltod, sodass dies die unter pharmakologischer PHGDH-Inhibition gewonnenen Daten bestätigte.

Umgekehrt zeigten genetisch veränderten Zellen, die durch eine induzierbare Überexpression der PHGDH charakterisiert waren, eine verminderte LDH-Freisetzung sowie eine erhöhte NADPH/$NADP^+$-Ratio passend zum vermuteten protektiven Effekt der PHGDH-Aktivität unter Bedingungen des Tumormikromilieus.

Diskussion

Unsere Ergebnisse bestätigen eine wichtige Bedeutung des Serin-Metabolismus als Regulator der Redox-Homöostase und des Tumorzellüberlebens unter Bedingungen des Mikromilieus des Glioblastoms.

Die durch Hypoxie hervorgerufene Induktion von PHGDH und der mitochondrialen SHMT2, nicht aber der zytosolischen SHMT1, weist darauf hin, dass der Serin-Metabolismus und insbesondere die mitochondriale Serin-Verstoffwechslung von Glioblastomzellen als Adaptationsmechanismus an widrige Wachstumsbedingungen genutzt wird. Dies bekräftigt ähnliche Ergebnisse zu Hypoxie-induzierter Hochregulation von Enzymen des Serinstoffwechsels in Tumorzellen[24,25].

Darüber hinaus vermittelte eine durch Transfektion gesteigerte PHGDH-Expression unter Bedingungen des Tumormikromilieus passend dazu protektive Effekte über eine Regulation der Redox-Homöostase.

In den Wachstumsanalysen zeigte sich ferner die Bedeutung der Serinverfügbarkeit sowohl durch Serinimport als auch durch endogen synthetisiertes Serin auf das Tumorzellwachstum. Passend zu diesen Ergebnissen konnte bereits gezeigt werden, dass eine serinfreie Diät oder PHGDH-Inhibition das Wachstum von Brustkrebs- und Melanomzellen reduziert[15,14]. Außerdem zeigte sich in weiteren *in vivo* Untersuchungen zum einen ein positiver Effekt auf das Überleben durch Serin- und Glycin-freie Diät in einem Kolonkarzinom-Modell[26]. Zum anderen wurden auch antitumorale Effekte durch den Einsatz von PHGDH-Inhibitoren in Untersuchungen zu Brustkrebs sowie hepatozellulären und renalen Karzinomen beobachtet. Hierbei zeigten die verwendeten Inhibitoren im Mausmodell eine gute Blut-Hirn-Schranken-Gängigkeit bei gleichzeitig niedriger Toxizität.[27-29]

Ferner untersuchten wir die Bedeutung des Serin-Metabolismus unter den extremen Bedingungen des Glioblastom-Mikromilieus. Dabei zeigte sich, dass PHGDH-Inhibition Hypoxie-induzierten Zelltod vermutlich über eine Störung der Redox-Homöostase induzieren kann. Dieser Effekt konnte durch Versuche mit Zellen mit stabiler Gensuppression der PHGDH bestätigt werden.

Zusammenfassend unterstreichen unsere Ergebnisse die Bedeutung des Serinmetabolismus im Glioblastom. Dabei scheint eine Hochregulation der Enzyme, insbesondere des mitochondrialen Serinstoffwechsels, einen relevanten Adaptationsmechanismus an die widrigen Bedingungen des Tumormikromilieus darzustellen. Dies könnte pharmakologisch genutzt werden, um sowohl das Tumorzellwachstum zu reduzieren als auch über eine Störung der zellulären Redox-Homöostase Tumor-Zelltod unter widrigen Bedingungen des Mikromilieus zu erleichtern.

Somit stellt der Serinmetabolismus ein interessantes neues Therapieziel für das Glioblastom dar.

Übersicht der Publikation

Die hier vorgestellte Publikation wurde am 17. März 2020 im British Journal of Cancer unter dem Titel “Serine-dependent redox homeostasis regulates glioblastoma cell survival” veröffentlicht.

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ARTICLE
Cellular and Molecular Biology

Serine-dependent redox homeostasis regulates glioblastoma cell survival

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BACKGROUND: The amino acid serine is an important substrate for biosynthesis and redox homeostasis. We investigated whether glioblastoma (GBM) cells are dependent on serine for survival under conditions of the tumour microenvironment.
METHODS: Serine availability in GBM cells was modulated pharmacologically, genetically and by adjusting serine and glycine concentrations in the culture medium. Cells were investigated for regulation of serine metabolism, proliferation, sensitivity to hypoxia-induced cell death and redox homeostasis.
RESULTS: Hypoxia-induced expression of *phosphoglycerate dehydrogenase (PHGDH)* and the mitochondrial *serine hydroxymethyltransferase (SHMT2)* was observed in three of five tested glioma cell lines. Nuclear factor erythroid 2-related factor (Nrf) 2 activation also induced *PHGDH* and *SHMT2* expression in GBM cells. Low levels of endogenous PHGDH as well as *PHGDH* gene suppression resulted in serine dependency for cell growth. Pharmacological inhibition of PHGDH with CBR-5884 reduced proliferation and sensitised cells profoundly to hypoxia-induced cell death. This effect was accompanied by an increase in reactive oxygen species and a decrease in the NADPH/NADP$^+$ ratio. Similarly, hypoxia-induced cell death was enhanced by *PHGDH* gene suppression and reduced by *PHGDH* overexpression.
CONCLUSIONS: Serine facilitates adaptation of GBM cells to conditions of the tumour microenvironment and its metabolism could be a plausible therapeutic target.

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BACKGROUND

Glioblastoma (GBM) is the most common primary CNS malignancy in adults with a dismal prognosis.[1] Multimodal therapeutic approaches including tumour resection, radiochemotherapy and tumour treating fields yield overall survival times of only about 21 months[2,3] and new treatment approaches are urgently needed.

Hypoxia is a common feature of the microenvironment of GBMs[4–7] caused by an imbalance of tumour growth and vascular supply.[8,9] Reactive oxygen species (ROS) are common by-products of aerobic metabolism primarily originating from mitochondria due to an incomplete reduction of oxygen in the electron transport chain.[10] ROS levels can be enhanced by elevated metabolic activity, hypoxia or chemotherapy.[11–13] Excessive ROS production can lead to the depletion of reducing substrates, ultimately resulting in oxidative damage and cytotoxicity.[14]

Reprogramming energy metabolism has more recently been acknowledged as a hallmark of cancer.[15] One well-known and extensively studied phenomenon of an altered cancer metabolism is aerobic glycolysis or the so-called Warburg effect. This describes the preferentially glucose metabolism via glycolysis without subsequent entry of substrates into the citric acid cycle despite the availability of oxygen resulting in a potential waste of energy.[16] Another metabolic pathway that has recently attracted attention in cancers is the serine synthesis pathway (SSP). In certain cancer types, such as breast cancer, melanoma and non-small cell lung cancer, the central enzymes of serine metabolism are upregulated and high SSP activity defines a more aggressive tumour subtype with worse prognosis.[17,18]

Besides its function as a synthesis substrate for proteins and lipids,[19,20] serine contributes to the one-carbon pool (1CM) to promote nucleotide synthesis.[19] Additionally, 1CM is a source for NADPH production via methylenetetrahydrofolate dehydrogenase (MTHFD), which catalyses the conversion of methylenetetrahydrofolate (MTHF) and NADP$^+$ to formyl-tetrahydrofolate (formyl-THF) and NADPH.[21] NADPH increases the cellular antioxidative capacity by regenerating the cellular pool of reduced glutathione and thioredoxin.[22]

Apart from its import via amino acid transporters[23,24] serine can be synthesised de novo from the glycolytic intermediate 3-phosphoglycerate (3-PG).[25] This first and rate limiting step to divert substrates from glycolysis to serine synthesis is catalysed by 3-PG dehydrogenase (PHGDH).[26] Focal amplifications of the *PHGDH* gene have been described in breast cancer and melanoma.[18,27] Recently, it has been demonstrated for these two entities that expression of

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PHGDH is a relevant factor for tumour cell proliferation when serine supply is limited.[28] In gliomas, PHGDH expression increases with WHO grade and silencing of *PHGDH* leads to reduced GBM cell proliferation and invasion.[29] Lately, a novel selective small-molecule inhibitor of PHGDH, CBR-5884, has been identified.[30] Serine hydroxymethyltransferases (SHMTs) catalyse the conversion of serine to glycine and vice versa.[31] SHMT1, the cytoplasmatic isoform, does not significantly contribute to the production of glycine, whereas SHMT2, the mitochondrial isoform, is an important source of glycine in proliferating cells.[32,33] In GBM, pseudopallisading cells surrounding necrotic regions express high levels of SHMT2 and glycine decarboxylase (GLDC). In those cells, SHMT2 reduces oxygen consumption to adapt to microenvironmental conditions.[34]

In this project we modulated serine availability and SSP enzymatic activity under conditions mirroring the GBM microenvironment. Serine- and glycine-free culture medium as well as CBR-5884 were used to limit import as well as endogenous serine production. We analysed a panel of glioma cell lines for basal expression of key SSP enzymes, propensity to serine synthesis and dependence on exogenous serine supplementation. Furthermore, expression of SSP enzymes, as well as cell survival, oxidative stress and NADPH production, were investigated under starvation conditions. We report that inhibition of SSP activity sensitises GBM cells to hypoxia-induced cell death by increasing reactive oxygen species.

METHODS

Reagents, cell lines and culture conditions

All reagents not specified were purchased from Sigma (St. Louis, MO, USA). CBR-5884, a PHGDH inhibitor, and RA 839, a Nrf2 (nuclear factor erythroid 2-related factor) activator, were purchased from Tocris (Bristol, UK). LNT-229, LN-308, LN-428 and G55 cells have been described.[35] LNT-229 and LN-308 cells were a kind gift from N. de Tribolet (Lausanne, Switzerland), G55 cells were a kind gift from Manfred Westphal and Kathrin Lamszus (Hamburg), LN-428 cells and LN-464 cells were a kind gift from Monika Hegi (Lausanne). MDA-MB-231 and MDA-MB-464 cells were a kind gift from Winfried Wels (Frankfurt, Germany).

Wildtype cell lines were maintained as described.[35] For experiments glycine- and serine-free DMEM (US Biological Life Sciences, catalog no. D9800-03/D9802-01, Salem, MA, USA) was supplemented with glucose or serine as indicated. FCS included serine and its supplementation yielded serine concentrations of 20 µM (in comparison to 400 µM when serine was replenished). pLKO.1- and pTetOne-transfected cells were maintained in medium with 2 µg/ml puromycin. 0.1 µg/ml doxycycline was added to induce gene expression of PHGDH from pTetOne-transfected cells. To compare sub cell lines equal cell densities were confirmed by crystal violet (CV) staining as described.[36]

Generation of PHGDH gene suppressed and PHGDH overexpressing cells

The pLKO.1 plasmid (Sigma, Clone-ID: TRCN 00000 28532) was used to mediate stable shRNA-mediated gene suppression of *PHGDH*. Control cells were transfected with a pLKO.1 plasmid with a non-targeting shRNA sequence (Addgene, catalog no. 1864, Watertown, MA, USA). Attractene (Qiagen, Hilden, Germany) was used for transfection. LNT-229 pTetOne PHGDH cells have been described.[37] Expression of *PHGDH* in the cell pool and single cell clones was quantified by qPCR after incubation with or without doxycycline. For further analysis one single cell clone with a 14-fold higher expression of *PHGDH* compared to control was used.

Induction of hypoxia

Hypoxia was induced as previously described.[36,38,39] Briefly, 0.1% oxygen was induced by incubation in GasPak™ pouches for anaerobic culture (Becton-Dickinson, Heidelberg, Germany).[36,38] Oxygen deprivation of 1% as well as 5% oxygen was induced in a Labotect incubator (Goettingen, Germany) as described.[39]

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR) analysis

The qPCR protocol employed has already been described.[35] Primer pairs are listed in the supplement (Suppl. Table 1). 18S and *SDHA* were both used as housekeeping genes for normalisation.

Immunoblot analysis

Immunoblot was performed as recently described.[35] Membranes were probed with antibodies to PHGDH (Santa Cruz Biotechnology, Dallas, TX, USA), SHMT1, SHMT2 (Atlas Antibodies, Bromma, Sweden) or actin (Santa Cruz Biotechnology, Dallas, TX, USA). The secondary anti-mouse and anti-goat antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The secondary anti-rabbit antibody was purchased from Jackson ImmunoResearch (Cambridgeshire, UK).

Amino acid measurements by LC-MS/MS

Amino acid measurements by LC-MS/MS was performed as recently described.[40] A detailed protocol is included in the supplement (Supplementary Methods).

Cell density and cell viability assays

Cell density measurement by crystal violet (CV) staining as well as cell viability measurement by lactate dehydrogenase (LDH) release assay with the Cytotoxicity Detection Kit (LDH) (Roche, Mannheim, Germany) have already been described.[35]

Reactive oxygen species measurement

Reactive oxygen species analysis was also performed as described previously.[35]

NADPH/NADP$^+$ measurement

NADPH and NADP$^+$ were measured with a luminescence-based assay (NADP/NADPH-Glo assay kit, Promega, Madison, WI, USA) according to the manufacturer's protocol.

Statistical analysis

Quantitative data are expressed as indicated including standard deviation (S.D.). *P*-values were derived from two-tailed student's *t*-tests. Values of $P > 0.05$ were considered not significant (n.s.). Values of $P < 0.05$ and $P < 0.01$ were considered significant and highly significant (Excel, Microsoft, Seattle, WA, USA).

RESULTS

Expression of key enzymes of serine metabolism varies between different glioma cell lines

mRNA expression and protein levels of key enzymes of serine metabolism were investigated in a panel of glioma cell lines. Breast cancer cell lines with reportedly low (MDA-MB-231) and high (MDA-MB-468) PHGDH expression[18] were analysed for comparison (Fig. 1a). GBM cell lines displayed a broad spectrum of PHGDH expression: LN-308 and LN-428 showed low expression comparable to PHGDH expression in MDA-MB-231 cells (Fig. 1a). In contrast G55 and LN-464 had almost similar PHGDH expression levels as MDA-MB-468 (Fig. 1a). LNT-229 had intermediate PHGDH levels (Fig. 1a). SHMT1 and 2 expression levels varied only moderately between the tested glioma cell lines (Fig. 1b).

PHGDH and *SHMT2* but not *SHMT1* are upregulated under hypoxic conditions in LN-308, LNT-229 and G55 cells

An upregulation of SSP enzymes in hypoxia has previously been found in breast cancer cells.[41] In line with that, hypoxia led to an

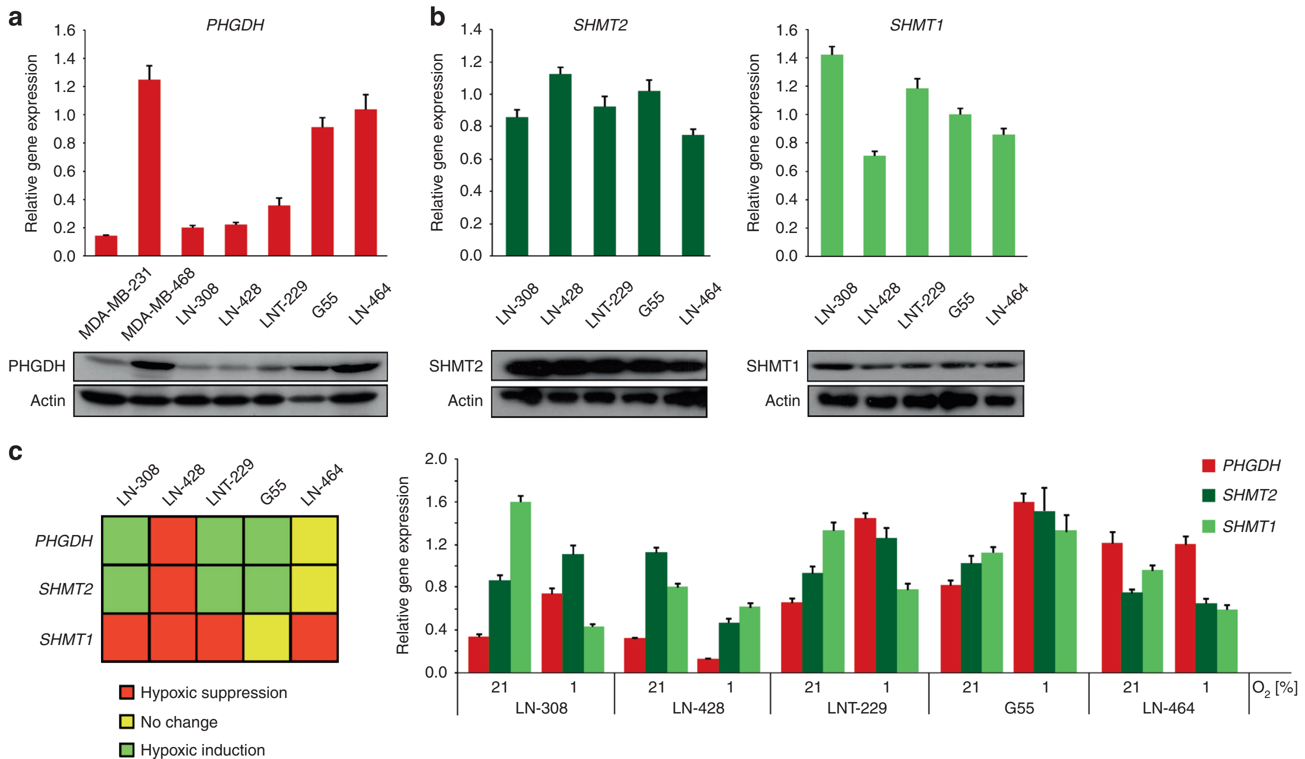

Fig. 1 Expression of key enzymes of SSP under normoxic and hypoxic conditions. a–b Gene expression (upper panel) and protein levels (lower panel) of the SSP enzymes PHGDH (**a**), SHMT1 and 2 (**b**) in breast cancer (MDA-MB-231 and MDA-MB-468) and glioma (LN-308, LN-428, LNT-229, G55 and LN-464) cell lines were investigated under standard conditions (DMEM containing 10% FCS and 25 mM glucose under normoxia) by qPCR and immunoblot. Values are normalised to *18 S* as well as *SDHA* housekeeping gene expression ($n = 3$, mean ± SD). Cellular lysates were analysed by immunoblot with antibodies for PHGDH, SHMT1, SHMT2 and actin. **c** Gene expression of the SSP enzymes *PHGDH*, *SHMT1* and *2* in glioma cell lines were investigated under starvation conditions (24 h in serum-free medium and 1% oxygen (O_2)). Gene expression was measured by qPCR. Values are normalised to *18 S* as well as *SDHA* housekeeping gene expression ($n = 3$, mean ± SD). Significant gene induction (*$p < 0.05$ or **$p < 0.01$) is illustrated by green boxes, significant gene suppression (*$p < 0.05$ or **$p < 0.01$) is illustrated by red boxes and no significant change in gene expression is illustrated by yellow boxes.

upregulation of *SHMT2* and *PHGDH* expression in MDA-MB-231 und MDA-MB-468 cells while *SHMT1* expression was not induced or even reduced under hypoxia (Suppl. Fig. 1A). To investigate a potential adaption of GBM cell SSP under deprivation conditions, expression levels of *PHGDH*, *SHMT1* and *2* were tested under hypoxic conditions with 1% hypoxia as already tested for breast cancer cell lines[41] (Fig. 1c). An upregulation of *PHGDH* and *SHMT2* was also observed in LN-308, LNT-229 and G55 cells. However, *PHGDH* and *SHMT2* levels were unchanged or reduced in LN-464 or LN-428 cells. *SHMT1* levels were unchanged or reduced in all tested cell lines.

As areas of GBMs can exhibit levels of profound hypoxia as low as 0.1% oxygen[42] and distinct areas of solid tumours display oxygen concentrations between 5% oxygen[43–47] and 0.1% oxygen,[48,49] we also investigated gene expression of SSP enzymes under 0.1 and 5% oxygen (Suppl. Fig. 1B). An upregulation of *PHGDH* was also observed in LN-308 and LNT-229 cells under 0.1% oxygen. *SHMT2* was upregulated in LN-308 and G55 cells under 0.1% oxygen. *SHMT1* gene expression was suppressed in all tested cell lines, except LN-308 under 0.1% oxygen. Under 5% oxygen only a slight induction of *SHMT2* was observed in LN-308 and LNT-229 cells. *PHGDH* gene expression was not induced under 5% oxygen in all tested cell lines. On the contrary, LN-428 and LN-464 even showed a gene suppression of *PHGDH* under 5% oxygen. In contrast to the published results of breast cancer cells[41] the hypoxia-induced upregulation of *SHMT2* was only affected by a double gene-suppression of HIF-1α and HIF-2α in GBM cells whereas a single HIF-1α or HIF-2α gene-suppression as well as a double gene-suppression of HIF-1α and HIF-2α did not prevent induction of *PHGDH* under hypoxia (Suppl. Fig. 2A). *SHMT1* expression was unaffected by hypoxia (Suppl. Fig. 2A). A regulation of *PHGDH* and *SHMT2* by the transcription factor Nrf2 has recently been reported in non-small cell lung cancer cells.[17] Similarly, we found an induction of *PHGDH* and *SHMT2* but not *SHMT1* by the Nrf2 activator RA 839 in LNT-229 and G55 cells (Suppl. Fig. 2B). Expression levels of the Nrf2-targets *heme oxygenase 1 (HO-1)* and *thioredoxin 1 (TXN-1)* are shown as indicators for Nrf2 activation by RA 839 (Suppl. Fig. 2B).

Serine availability is required for tumour cell growth

To investigate the effect of serine deprivation on tumour cell proliferation we targeted three potential sources of serine: (i) serine import, (ii) serine synthesis from glycine *via* SHMT1 and 2 and (iii) de novo synthesis via PHGDH by employing glycine- and serine-free DMEM as well as the novel small-molecule PHGDH inhibitor CBR-5884.[30] The combination of glycine- and serine-free DMEM with 60 µM CBR-5884 resulted in a significantly reduced intracellular serine and glycine level in comparison to vehicle in G55 cells (Fig. 2a). In contrast, asparagine and tyrosine (used as a control) levels were not affected by CBR-5884. Under serum containing culture conditions serine and glycine deprivation alone led to a significant decrease of tumour cell proliferation in LN-308 cells with an intrinsically low PHGDH expression. Proliferation of cell lines with intermediate (LNT-229) or high (G55) PHGDH expression was not affected by serine and glycine restriction under serum containing conditions (Fig. 2b, upper panel). Under serum-free conditions cell proliferation was inhibited in LNT-229

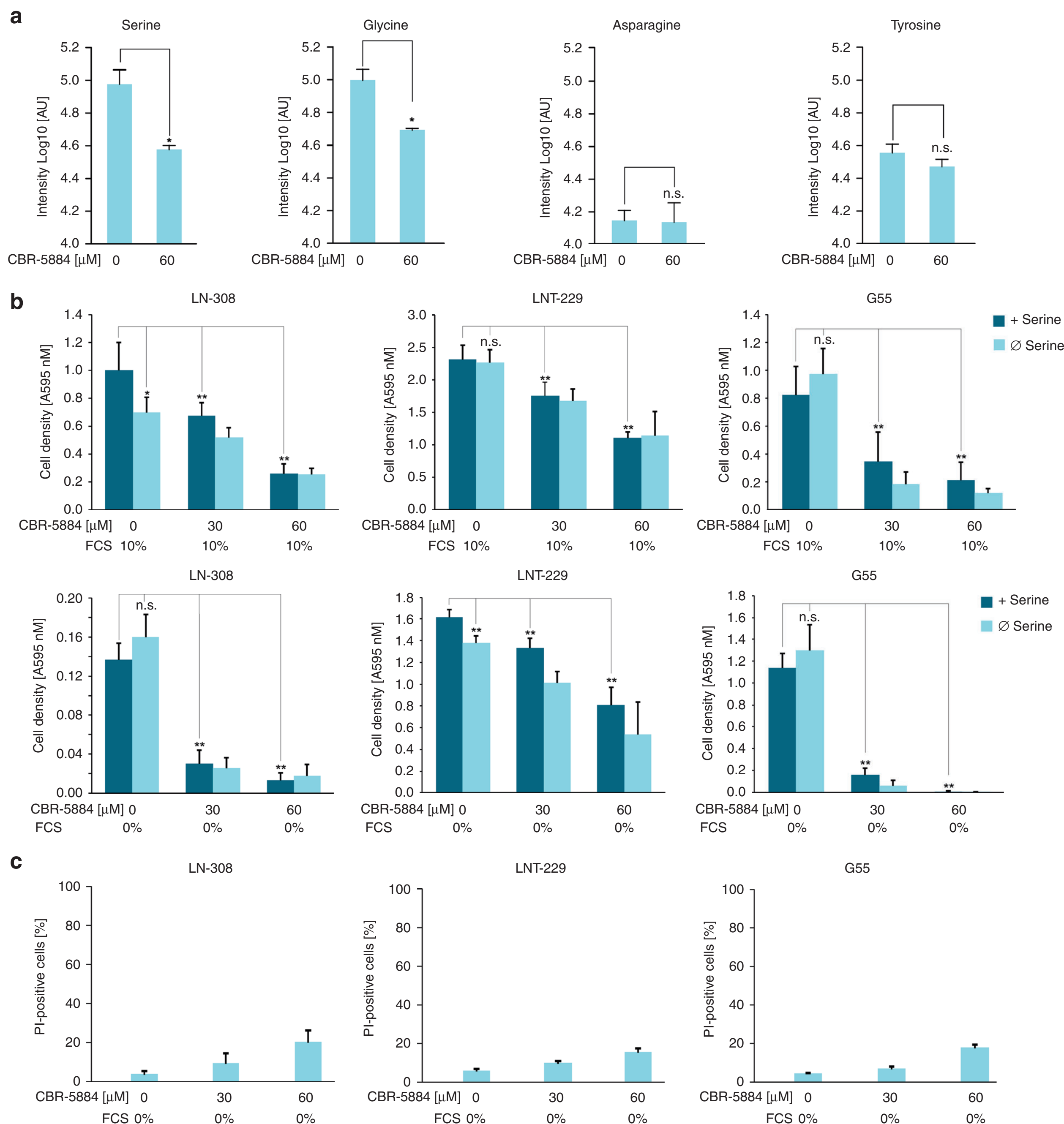

Fig. 2 Effects of serine availability on tumour cell growth. a G55 cells were incubated in serum-free medium without glucose restriction (25 mM) depleted for glycine and serine with vehicle or 60 µM CBR-5884 as indicated for 24 h. Serine, glycine, asparagine and tyrosine levels were measured by LC-MS/MS ($n = 3$, mean ± SD, n.s. not significant, *$p < 0.05$). **b** LN-308, LNT-229 and G55 cells were incubated in DMEM (25 mM glucose) depleted for glycine and serine with or without CBR-5884 as indicated. Serine was replenished as indicated, with (upper panel) and without (lower panel) 10% FCS. Cells were incubated for 4 days. Cell density was measured by crystal violet staining at the beginning of cultivation and at 4 days ($n = 6$, mean ± SD, n.s. not significant, *$p < 0.05$, **$p < 0.01$). C, LN-308, LNT-229 and G55 cells were incubated in serum-free DMEM depleted for glycine and serine with or without CBR-5884 as indicated. Cell death was quantified by PI-uptake after 4 days ($n = 3$, mean ± SD).

cells by serine and glycine deprivation but not in G55 cells. LN-308 cells showed almost no proliferation under serum-free conditions (Fig. 2b, lower panel). PHGDH inhibition with CBR-5884 inhibited tumour cell growth in all tested cell lines in a dose-dependent manner. This effect was even stronger under serum-free culture conditions (Fig. 2b, lower panel). In a parallel PI-FACS analysis toxicity of CBR-5884 under serum-free, serine- and glycine-free conditions was analysed and revealed only mild to moderate cell death under increasing CBR-5884 concentrations (Fig. 2c).

Serine restriction sensitises for hypoxia-induced cell death and impairs redox homeostasis

We next investigated whether intracellular serine levels influence tumour cell survival under conditions of the tumour microenvironment. LN-308 (low PHGDH expression) and LNT-229 (moderate PHGDH expression) showed increased cell death under serine and glycine deprivation alone (Fig. 3a). G55 (high PHGDH expression) cells were unaffected by serine and glycine deprivation. CBR-5884 increased hypoxia-induced cell death in all tested cell lines. Similar

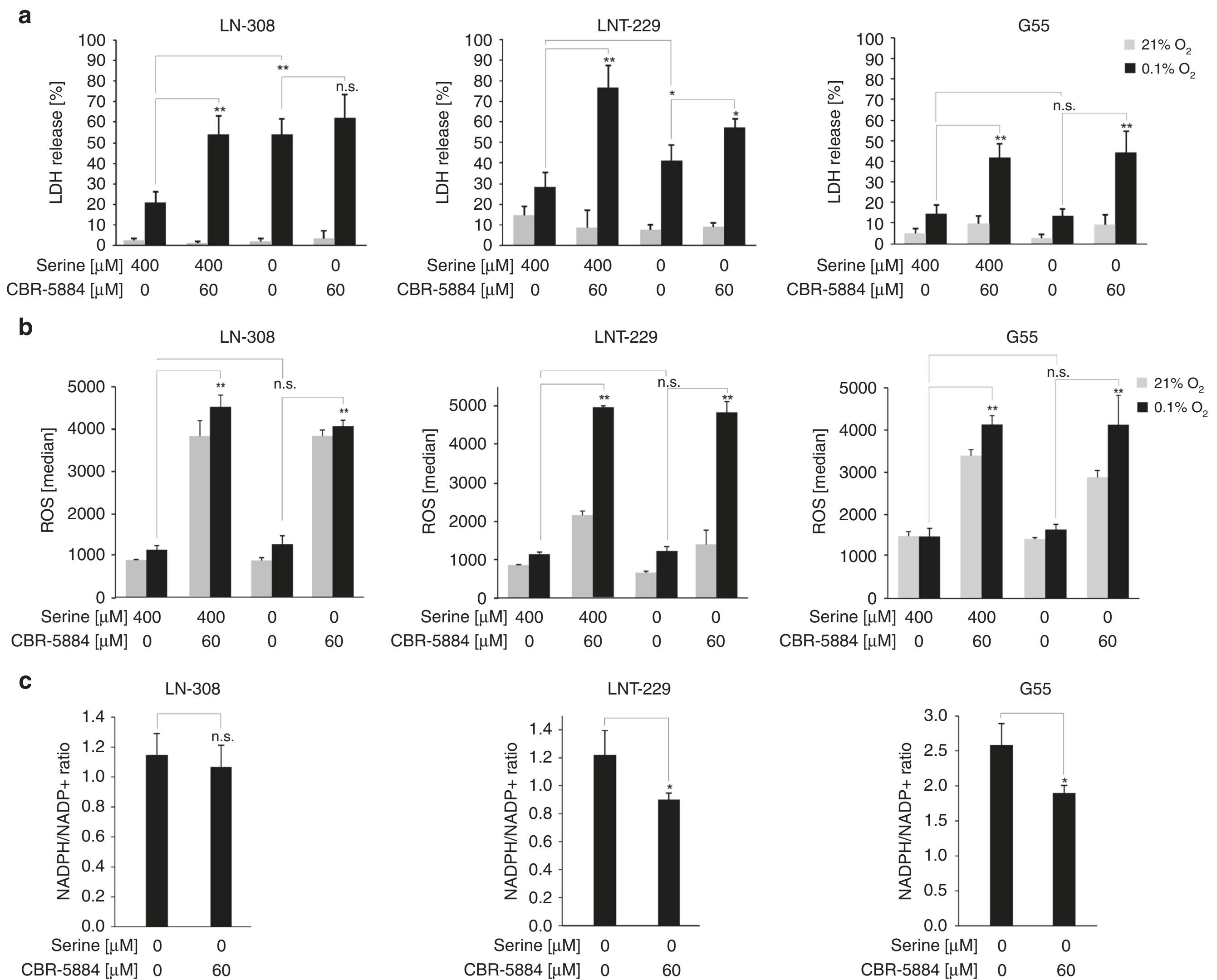

Fig. 3 Serine restriction sensitises for hypoxia-induced cell death and increases intracellular ROS levels. a–c LN-308, LNT-229 and G55 cells were exposed to glucose restricted (2 mM glucose) serum- and glycine-free DMEM under normoxic or hypoxic (0.1% oxygen (O_2)) conditions with or without CBR-5884 as indicated. Serine was replenished as indicated. **a** Cell death was quantified by LDH-release ($n = 4$, mean ± S.D., n.s. not significant, *$p < 0.05$, **$p < 0.01$). **b** Reactive oxygen species (ROS) levels were measured by H2DCFDA-FACS ($n = 3$, mean ± SD, n.s. not significant, **$p < 0.01$). **c** Analysis of NADPH/$NADP^+$ ratios was performed by a luminescence-based assay ($n = 3$, mean ± SD, n.s. not significant, *$p < 0.05$).

results were obtained by PI-FACS analysis (data not shown). In line with these results ROS levels were increased under treatment with CBR-5884 in all tested cell lines (Fig. 3b). Measurement of the NADPH/$NADP^+$ ratio showed that PHGDH inhibition led to a significant decrease of the NADPH/$NADP^+$ ratio in cells with moderate (LNT-229) or high (G55) PHGDH expression (Fig. 3c). However, in LN-308 cells (low PHGDH expression) only a trend towards a lower NADPH/$NADP^+$ ratio could be observed.

PHGDH gene suppression sensitises human GBM cells to hypoxia-induced cell death

To further confirm the robustness of the observed phenotype and rule out off-target effects of CBR-5584, cells with gene suppression of *PHGDH* were generated. G55 cells were chosen due to their high endogenous PHGDH level. QPCR and immunoblot confirmed stable gene suppression of *PHGDH* (PHGDHsh) compared to control cells (NTsh) (Fig. 4a). Cell proliferation was impaired by serine and glycine withdrawal under serum-free and serum containing culture conditions only in G55 PHGDHsh cells mimicking the phenotype of cells with low PHGDH expression (Fig. 4b). Furthermore, G55 PHGDHsh cells displayed enhanced sensitivity to hypoxia-induced cell death coherent with results obtained with pharmacological PHGDH inhibition (Fig. 4c). However, serine withdrawal did not affect sensitivity to hypoxia-induced cell death in PHGDHsh cells (Fig. 4c).

PHGDH overexpression protects human GBM cells from hypoxia-induced cell death

LNT-229 cells that inducibly overexpress *PHGDH* were generated (LNT-229 pTetOne PHGDH). Gene induction by doxycycline was confirmed by qPCR and immunoblot (Fig. 4d). In contrast to PHGDHsh cells, LNT-229 pTetOne PHGDH cells displayed protection from hypoxia-induced cell death when *PHGDH* was induced (Fig. 4e). In addition, *PHGDH* induction increased the NADPH/$NADP^+$ ratio (Fig. 4f).

DISCUSSION

Our results describe serine metabolism as an important regulator of cellular redox homeostasis and tumour cell survival under conditions of the glioma microenvironment.

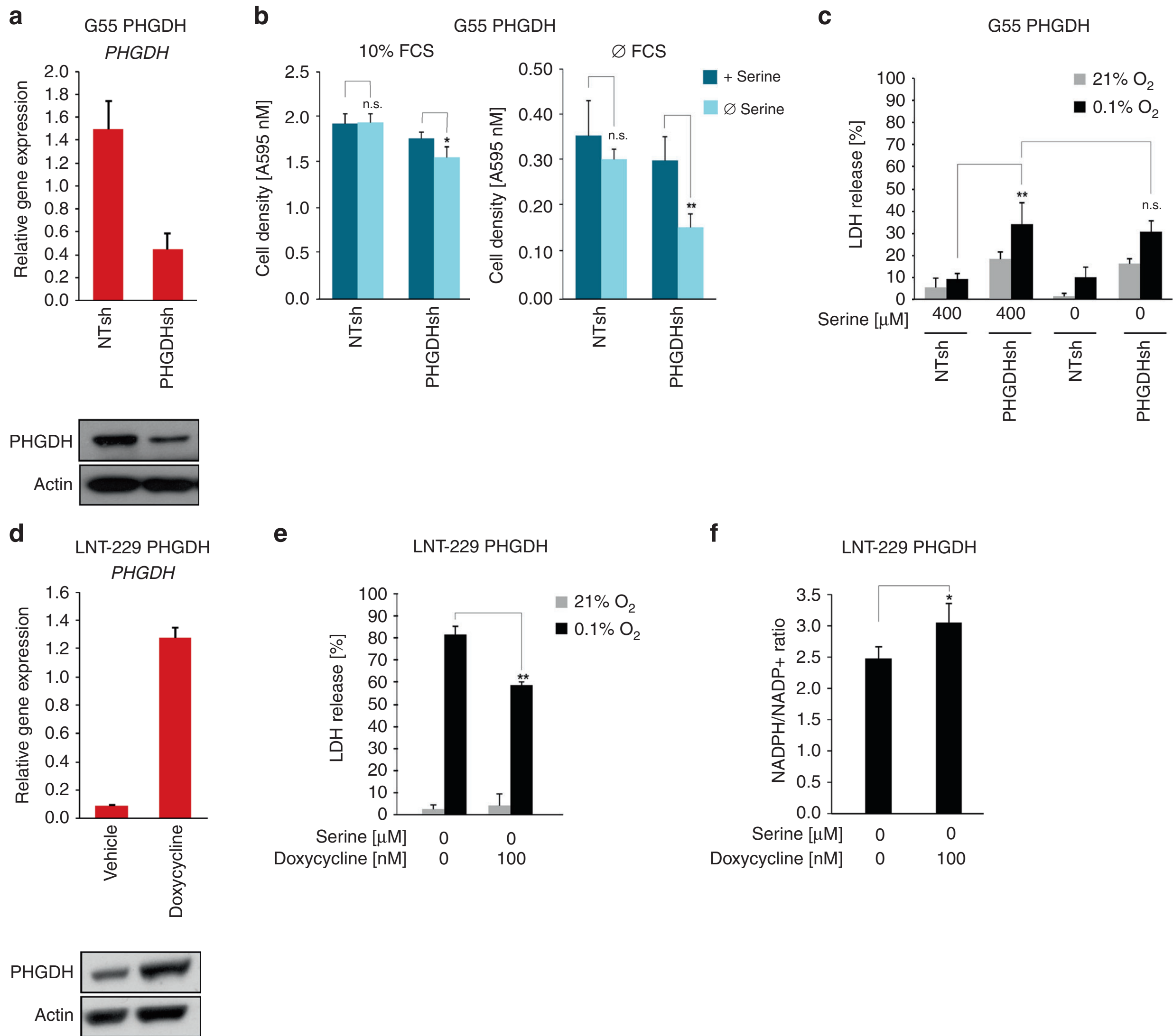

Fig. 4 ***PHGDH* gene suppression sensitises while PHGDH overexpression protects human GBM cells from hypoxia-induced cell death.** **a** G55 PHGDHsh and control cells (non-targeting sequence, NTsh) were analysed by qPCR and immunoblot. *PHGDH* gene suppression was confirmed. qPCR values are normalised to *18 S* as well as *SDHA* housekeeping gene expression ($n = 3$, mean ± SD). **b** G55 PHGDHsh and control cells were incubated in DMEM with or without 10% FCS as indicated. Medium was depleted for glycine and serine and serine was replenished where indicated. Cell density was measured by crystal violet staining at the beginning of cultivation and at 4 days ($n = 6$, mean ± S.D., n.s. not significant, $*p < 0.05$, $**p < 0.01$). **c** G55 PHGDHsh and control cells were exposed to glucose restricted (2 mM glucose) serum-free DMEM under normoxic or hypoxic (0.1% oxygen (O_2)) conditions. Serine was replenished as indicated. Cell death was quantified by LDH release ($n = 4$, mean ± S.D., n.s. not significant, $**p < 0.01$). **d** LNT-229 pTetOne PHGDH cells were cultured with vehicle or 0.1 µg/mL doxycycline for 24 h. *PHGDH* gene induction was confirmed by qPCR and immunoblot. qPCR values are normalised to *18 S* as well as *SDHA* housekeeping gene expression ($n = 3$, mean ± SD). E-F, LNT-229 pTetOne PHGDH cells were preincubated with or without 0.1 µg/ml doxycycline for 24 h and exposed to glucose restricted (2 mM glucose) serum- and serine-free DMEM with or without 0.1 µg/ml doxycycline under normoxic or hypoxic (0.1% oxygen (O_2)) conditions. **e** Cell death was quantified by LDH release ($n = 4$, mean ± S.D., $**p < 0.01$). ($n = 4$, mean ± S.D.) **f** Analysis of NADPH/NADP$^+$ ratios were performed by a luminescence-based assay ($n = 3$, mean ± SD, $*p < 0.05$).

The relevance of serine metabolism in GBM cells was first shown by demonstrating an induction of enzymes of SSP under hypoxic conditions that mirror the in vivo GBM situation. Our results on hypoxic upregulation of *PHGDH* and mitochondrial *SHMT2* (Fig. 1c, Suppl. Fig. 1B) emphasise the robustness of the phenomenon of a hypoxia dependent upregulation of SSP enzymes in cancer.[34,41] Furthermore, we could show that overexpression of PHGDH protected GBM cells from hypoxia-induced cell death (Fig. 4e) and sustained NADPH/NADP$^+$ ratios under starvation conditions (Fig. 4f). Our results affirm that high PHGDH levels protect GBM cells under most adverse conditions of the glioma microenvironment by maintaining redox homeostasis. Therefore, induction of SSP enzymes seems to serve as an adaptive response to adverse conditions of the tumour microenvironment. Vice versa, we could show for the first time that PHGDH inhibition with the new small-molecule inhibitor CBR-5884 sensitises GBM cells towards the conditions of the microenvironment, including glucose deprivation and severe hypoxia (Fig. 3a, Suppl. Fig. 3). This effect could be observed in all tested cell lines regardless of the PHGDH expression levels. Hypoxia-induced cell death under PHGDH inhibition was accompanied by an increase in intracellular ROS (Fig. 3b, Suppl. Fig. 3). Furthermore, for LNT-229 and G55 cells with moderate to high PHGDH expression levels, a decrease in the NADPH/NADP$^+$-ratio could also be observed after treatment with CBR-5884 (Fig. 3c, Suppl. Fig. 3).

Beyond that, PHGDH inhibition with CBR-5884 reduced cell proliferation in a dose-dependent manner in all tested cell lines regardless of the PHGDH expression levels (Fig. 2b). These results are in contrast to the data of the inhibitor's developers, who observed only growth inhibitory effects of CBR-5884 in cancer cell lines with high PHGDH expression levels and a high propensity for serine synthesis.[30] However, in this publication only concentrations up to 30 µM CBR-5884 were applied for cell proliferation assays, which is below the IC_{50} of 33 (±12) µM.[30]

Remarkably, GBM cells varied in their dependency on serine import. Serine deprivation alone showed only a significant reduction of cell growth and an increase in hypoxia-induced cell death in cells with low or moderate PHGDH expression (LN-308 and LNT-229) suggesting their dependency on import of extracellular serine (Figs. 2b, 3a). In contrast, G55 with higher PHGDH expression levels were less susceptible to sole serine starvation (Figs. 2b, 3a). Our results on growth propensity to serine import are in line with recent findings demonstrating that either increased PHGDH expression or increased serine supply provide a proliferative advantage in breast cancer and melanoma.[28] Corroborating these results, gene suppression of *PHGDH* rendered G55 cells susceptible to serine starvation under normoxia (Fig. 4b). In contrast, however, CBR-5884-mediated PHGDH inhibition did not influence the growth propensity of G55 cells to serine import (Fig. 2b). Furthermore, treatment with CBR-5884 as well as gene suppression of *PHGDH* in G55 did not lead to a sensitisation to serine withdrawal under hypoxic conditions (Figs. 3a, 4c). One reason for this discrepancy could be a potential contribution of other SSP enzymes or factors so that PHGDH inhibition alone is not sufficient to sensitise to serine withdrawal. Also, PHGDH inhibition could already induce a strong growth inhibition and sensitisation to hypoxia-induced cell death in G55 cells narrowing the potential of serine depletion for additional effects.

Taken together high intracellular serine levels seem to be favourable for tumour cells under stressful conditions of the glioma microenvironment. Both proliferation and survival were impaired by serine pathway inhibition under nutrient and oxygen deprivation. As serine and glycine are nonessential amino acids, one possible and feasible therapeutic approach could be a serine/glycine-free diet. In line with that, mice bearing colorectal xenograft tumours fed with a diet lacking serine and glycine displayed a reduction in tumour volume and a prolonged survival.[50] Moreover, several in vivo mouse experiments with PHGDH inhibitors alone or as a combined approach have shown positive antitumour effects in different tumour entities, such as breast cancer, renal cell carcinoma and hepatocellular carcinoma.[51–53] Our data indicate that a combination of a serine/glycine-free diet with PHGDH inhibition could be an even more effective approach. Besides inhibitors of PHGDH, recently inhibitors of SHMT1/2 have been described.[54] Serine depletion by diet and/or PHGDH inhibition might cause severe neurological side effects. In this respect *PHGDH* conditional knockout mice show mild microcephaly and forebrain atrophy.[55] However, effects of congenital gene depletion cannot be transferred to the setting of a mature human brain. Furthermore, in our settings PHGDH inhibition with CBR-5884 seemed to allow residual enzyme activity (Fig. 2a) while still impacting cell survival (Fig. 3a). Encouragingly, previous *in vivo* mouse experiments with PHGDH inhibitors demonstrated a tolerable toxicity profile with neither weight loss nor abnormal behaviour.[51–53] Therefore, further in vivo animal studies on neurological side effects as well as clinical trials on the efficacy and safety of the combination of serine/glycine deprivation with inhibition of the SSP, especially of PHGDH, are exciting future options for a serine-targeted therapeutic approach in cancer.

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AUTHOR CONTRIBUTIONS

A.L.L. and M.W.R. conceived the study; A.L.E., N.I.L., K.K., C.D. and A.L.L. carried out the experiments; A.L.E., N.I.L., K.K., C.D., C.M., J.P.S., M.W.R. and A.L.L. analysed the data; MWR, J.P.S. and A.L.L., coordinated the study, A.L.E. and A.L.L. wrote the manuscript; all authors helped drafting the manuscript and read and approved the final version.

ADDITIONAL INFORMATION

Ethics approval and consent to participate The human cell lines used in this study are all commercially or academically available and were not generated in the course of this study, and therefore no ethics approval was necessary for the cell line experiments. LNT-229 and LN-308 cells were a kind gift from N. de Tribolet (Lausanne, Switzerland), G55 cells were a kind gift from Manfred Westphal and Kathrin Lamszus (Hamburg), LN-428 cells and LN-464 cells were a kind gift from Monika Hegi (Lausanne). MDA-MB-231 and MDA-MB-464 cells were a kind gift from Winfried Wels (Frankfurt, Germany). LN-229 cells stably expressing a shRNA targeting *HIF-1α, HIF-2 α, HIF-1 + 2α* and control (*SIMA, homologue of HIF-1α in drosophila*), were kindly provided by C. Depner and T. Acker.

Consent to publish Not applicable.

Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. Supplementary information is available at the British Journal of Cancer's website.

Competing interests J.P.S. reports honoraria for lectures or advisory board participation or consulting or travel grants from Abbvie, Roche, Boehringer, Bristol-Myers Squibb, Medac, Mundipharma and UCB. All other authors declare no conflicts of interest. M.W.R. reports a research grant from UCB.

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SUPPLEMENTARY METHODS

Cell lines and culture conditions LN-229 cells stably expressing a shRNA targeting HIF-1α, HIF-2 α, HIF-1+2α and control (SIMA, homologue of HIF-1α in drosophila), were kindly provided by C. Depner and T. Acker. Lentiviral production was performed according to the BLOCK-iT™ Lentiviral Pol II miR RNAi Expression System using the plasmid pLenti6/V5-DEST (Invitrogen). Lentiviral infection was carried out using a multiplicity of infection of 60 cells. For selection of polyclonal stable transfected cells 10 μg/mL blasticidin was added to the culture medium.

Suppl. Table 1: Primer pairs for qRT-PCR analysis

	Fwd	Rev
18S	5'-CGGCTACCACATCCAAGGAA-3'	5'-GCTGGAATTACCGCGGCT-3'
SDHA	5'-TGGGAACAAGAGGGCATCTG-3'	5'-CCACCACTGCATCAAATTCATG-3'
PHGDH	5'-CTGCGGAAAGTGCTCATCAGT-3'	5'-TGGCAGAGCGAACAATAAGGC-3'
SHMT1	5'-CTGGCACAACCCCTCAAAGA -3'	5'-AGGCAATCAGCTCCAATCCAA-3'
SHMT2	5'-CCCTTCTGCAACCTCACGAC-3'	5'-TGAGCTTATAGGGCATAGACTCG-3'
HIF-1α	5'-GTCGGACAGCCTCACCAAACAGAGC-3'	5'-GTTAACTTGATCCAAAGCTCTGAG-3'
HIF-2α	5'-GCGCTAGACTCCGAGAACAT-3'	5'-TGGCCACTTACTACCTGACCCT-3'
CAIX	5'-AAGAAGAGGGCTCCCTGAAG-3'	5'-TAGCGCCAATGACTCTGGTC-3'
HO-1	5'-CTGCTCAACATCCAGCTCTTTG-3'	5'-AGTGTAAGGACCCATCGGAGA-3'
TXN-1	5'-ACGGTGATGCTGGCAATAGG-3'	5'-CTGGGGTGAGCTCCACCTTA-3'

Amino acid measurements by LC-MS/MS

Amino acid measurements by LC-MS/MS was performed as recently described (1). Cells were lysed using 300 µL of ice-cold 80 % methanol solution and cleared by centrifugation at 13000 rpm for 5 min at 4 °C. 30 µL of sample were mixed with 70 µL of 50 mM TEAB solution and 1 µL of TMT reagent (ThermoFisher Scientific, 0.8 mg TMT reagent resuspended in 41 µL anhydrous acetonitrile). Reactions were performed for 1 hour at room temperature and quenched by addition of hydroxylamine to a final concentration of 0.5 % and incubation for 15 min. Equal amounts of samples were pooled and diluted 1:100 in 0.1 % formic acid solution for LC-MS analysis.

As an internal standard an analytical standard mix of 17 amino acids (Sigma, catalog no. AAS18-5ML) was diluted 1:250 in water and labeling was performed as described above with TMT-0 reagent. 4 µL of labeled standard was mixed into sample multiplex before dilution.

4 µL of sample was separated on an Easy nLC II (ThermoFisher Scientific) and a 23 cm long, 75 µM ID fused-silica column, which has been packed in house with 3 µM C18 particles (ReproSil-Pur, Dr. Maisch), and kept at 50 °C using an integrated column oven (Sonation). Seperation was performed by a linear gradient from 1.8 85.5 % acetonitrile over 30 min and sprayed directly into an Orbitrap Fusion Lumos mass spectrometer using a nanoFlex ion source (ThermoFisher Scientific, Waltham, MA, USA) at a spray voltage of 2.6 kV. Full scan MS were acquired in range of 295 600 m/z at an orbitrap resolution of 120,000 at m/z 200 with a maximum injection time of 50 ms and an AGC target value of 6x105. Cycle time was set to 1.2 seconds and TMT-0 labelled amino acids were targeted for MS2 scans according to calculated masses. TMT-6 labelled amino acids were selected using a targeted mass difference of 5.0104 Da. To avoid repeated measures dynamic exclusion was set to 5 seconds. Ions were isolated for MS/MS with an isolation window of 0.4 Th, fragmented using high energy collisional dissociation

(normalized collision energy of 38) and analysed in the orbitrap with a resolution of 50,000, a maximum injection time of 86 ms and an AGC target value of 5x104.

TMT quantifications were manually extracted from averaged MS2 spectra using Freestyle 1.5 (ThermoFisher Scientific, Waltham, MA, USA).

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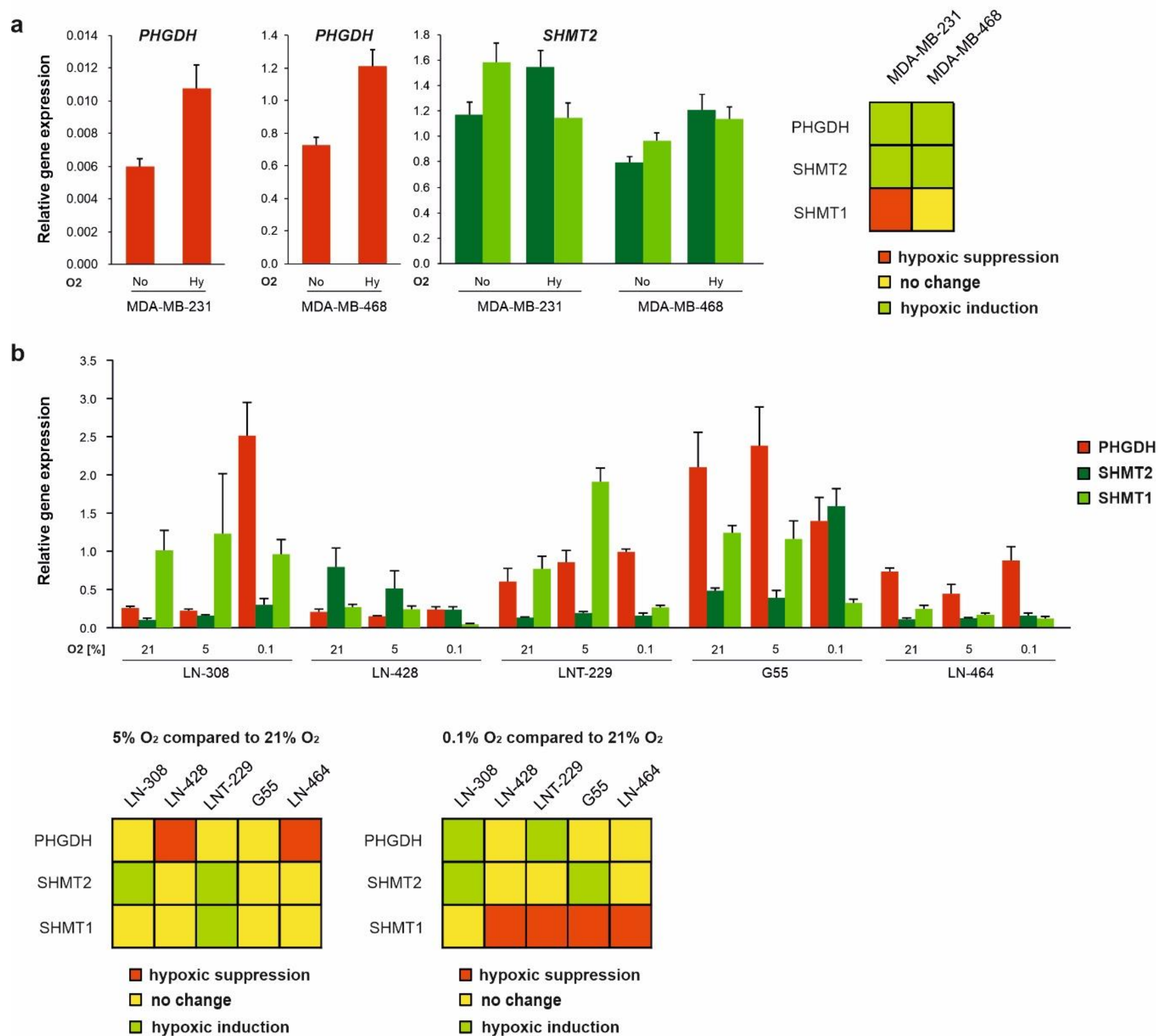
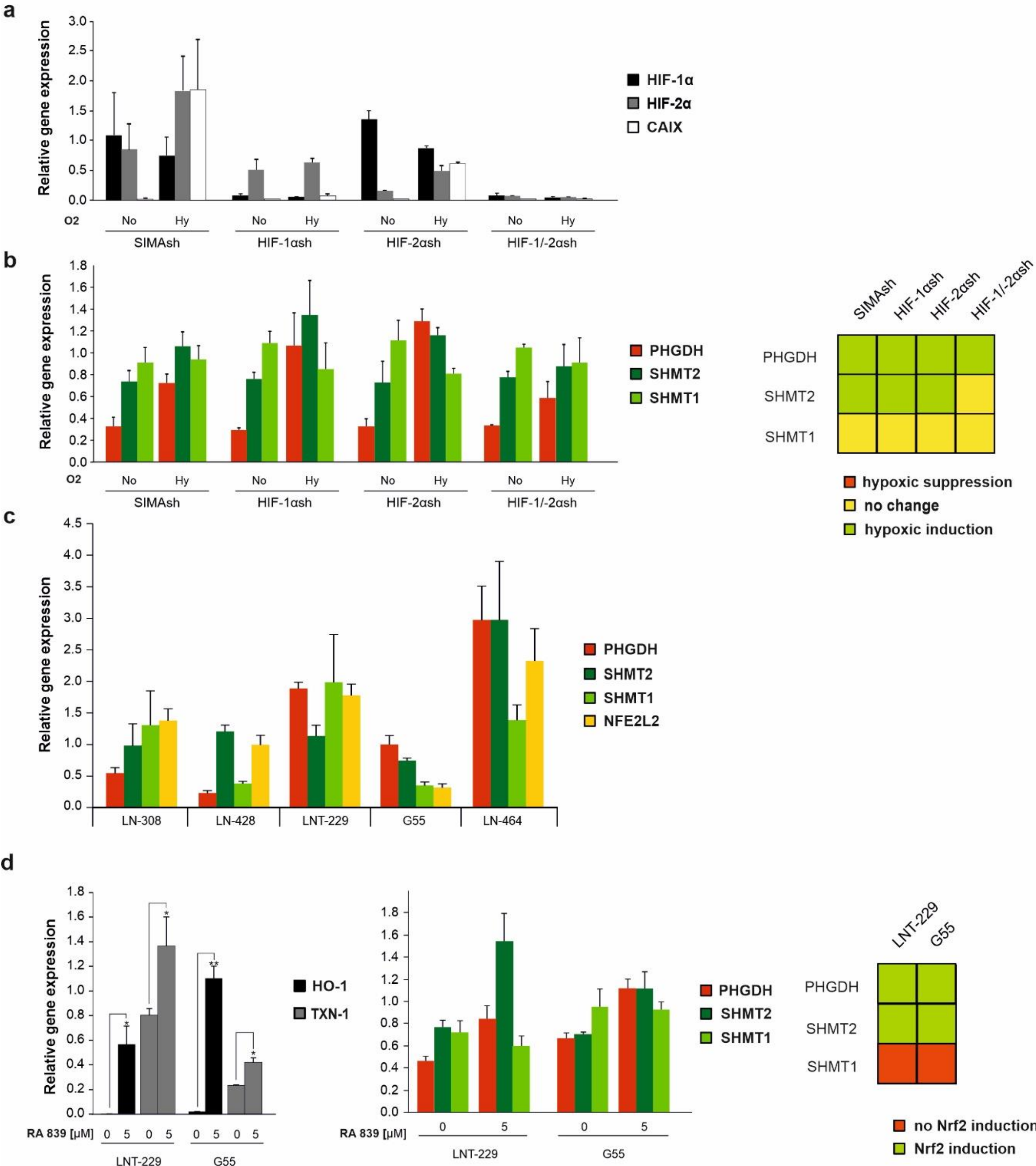


Supplementary Figure 1: PHGDH and SHMT2 are upregulated under hypoxic conditions

A, MDA-MB-231 and MDA-MB-468 breast cancer cells were exposed to serum-free DMEM under normoxic or hypoxic (1% oxygen) conditions. Gene expression of the SSP enzymes PHGDH, SHMT1 and 2 was investigated by qPCR. Values are normalized to 18S as well as SDHA housekeeping gene expression (n = 3, mean ± SD). Significant gene induction (*p<0.05 or **p<0.01) is illustrated by green boxes, significant gene suppression (*p<0.05 or **p<0.01) is illustrated by red boxes and no significant change in gene expression is illustrated by yellow boxes. B, glioma cells were exposed to serum-free DMEM under 21, 5 or 0.1% oxygen. Gene expression

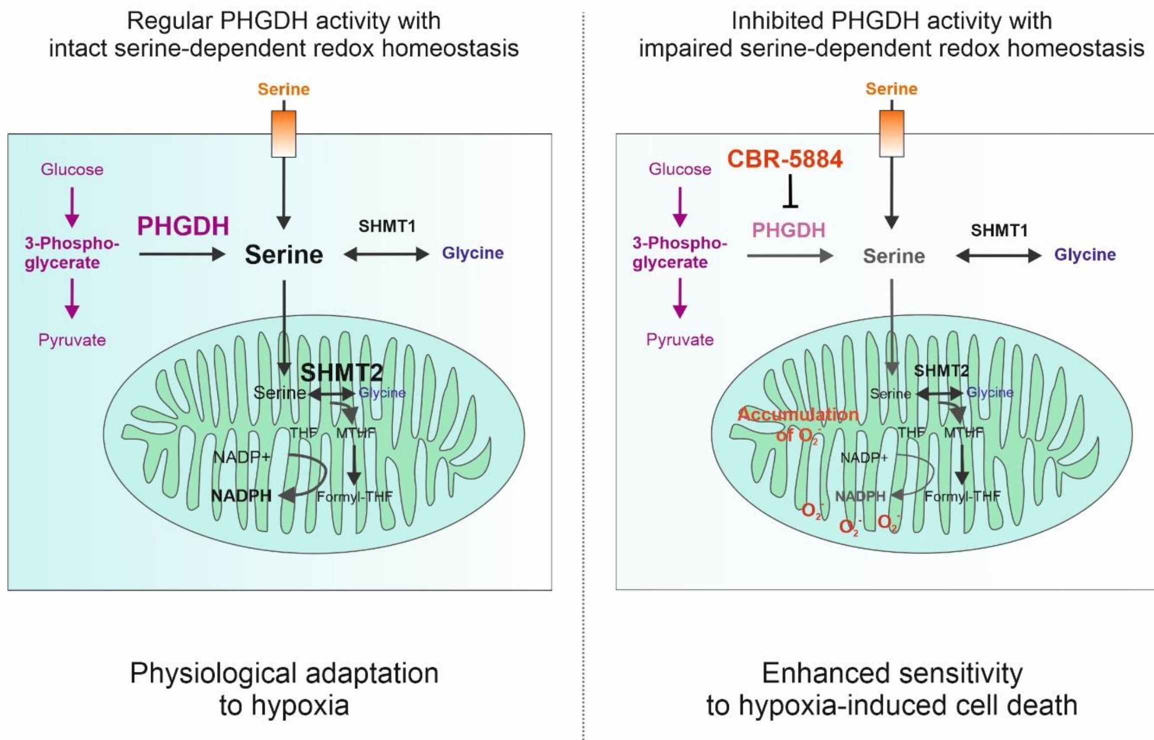
of the SSP enzymes PHGDH, SHMT1 and 2 was investigated by qPCR. Values are normalized to 18S as well as SDHA housekeeping gene expression (n = 3, mean ± SD). Significant gene induction (*p<0.05 or **p<0.01) is illustrated by green boxes, significant gene suppression (*p<0.05 or **p<0.01) is illustrated by red boxes and no significant change in gene expression is illustrated by yellow boxes.



Supplementary Figure 2: PHGDH and SHMT2 are upregulated under hypoxic conditions in a Nrf2 dependent manner

A, upper panel: LN-229 SIMAsh, HIF-1αsh, HIF-2α and HIF-1/-2α glioma cells were exposed to DMEM containing 10% FCS under normoxic or hypoxic (0.1% oxygen) conditions. Gene expression of HIF-1α, HIF-2α and the HIF target carbonic anhydrase IX (CAIX) was investigated by qPCR. Values are normalized to 18S as well as SDHA housekeeping gene expression (n = 3, mean ± SD). Lower panel: LN-229 SIMAsh, HIF-1αsh, HIF-2α and HIF-1/-2α cells were exposed to serum-free DMEM under normoxic or hypoxic (1% oxygen) conditions. Gene expression of the SSP enzymes PHGDH, SHMT1 and 2 was investigated by qPCR. Values are normalized to 18S as well as SDHA housekeeping gene expression (n = 3, mean ± SD). Significant gene induction (*$p<0.05$ or **$p<0.01$) is illustrated by green boxes and no significant change in gene expression is illustrated by yellow boxes. B, Gene expression of the SSP enzymes PHGDH, SHMT1 and 2 as well as HO-1 and TXN-1 in LNT-229 and G55 cells was investigated by qPCR under normoxic conditions in serum-free DMEM with or without 5 µM of the Nrf2 activator RA 839 for 6 h. Values are normalized to 18S as well as SDHA housekeeping gene expression (n = 3, mean ± SD, n.s. = not significant, *$p<0.05$, **$p<0.01$). For SSP enzymes significant gene induction (*$p<0.05$ or **$p<0.01$) is illustrated by green boxes and no significant induction is illustrated by yellow boxes.

Suppl. Fig. 3



Supplementary Figure 3: Schematic overview of metabolic changes in PHGDH impaired glioma cells

The PHGDH inhibitor CBR-5884 or PHGDH gene-suppression reduce intracellular serine levels in human GBM cells (right panel). A consequential decrease in the NADPH/NADP$^+$ ratio with elevated ROS levels ultimately triggers enhanced sensitivity to hypoxia-induced cell death.

Darstellung des eigenen Anteils

Die Idee zur Untersuchung des Serin-Metabolismus im Glioblastom stammt von Dr. Anna-Luisa Luger und PD Dr. Dr. Michael W. Ronellenfitsch. Ich führte eine umfassende Literaturrecherche durch und entwickelte in Rücksprache mit beiden zusammen einen Versuchsplan. Dr. Anna-Luisa Luger und Frau Dipl. Biochem. Nadja I. Lorenz arbeiteten mich in die Laborabläufe ein. Ich führte den Großteil der Experimente selbst durch. Dr. Anna-Luisa Luger und Nadja I. Lorenz unterstützten mich hierbei. Kevin Klann und Dr. Christian Münch führten am Institut für Biochemie mit von mir vorbereiteten Proben die Massenspektroskopie durch. Dr. Cornelia Depner stellte HIF-Knockdown-Zellen her, welche im Supplement vorkommen. Die gewonnenen Daten konnte ich mit allen Beteiligten besprechen und eventuelle Rückfragen klären. Dr. Anna-Luisa Luger und ich verfassten gemeinsam das Manuskript der vorliegenden Publikation. Alle Autoren kommentierten das Manuskript und stimmten der Veröffentlichung zu.

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Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

Die Bedeutung des Serin-Metabolismus für die Redox-Homöostase und das Überleben von Glioblastomzellen im Tumormikromilieu

in dem Zentrum der Neurologie und Neurochirurgie, Klinik für Neurologie, Funktionsbereich Neuroonkologie unter Betreuung und Anleitung von PD Dr. Dr. Michael Ronellenfitsch mit Unterstützung durch Dr. Anna-Luisa Luger und ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Vorliegende Ergebnisse der Arbeit wurden in folgendem Publikationsorgan veröffentlicht:

Anna L. Engel, Nadja I. Lorenz, Kevin Klann, Christian Münch, Cornelia Depner, Joachim P. Steinbach, Michael W. Ronellenfitsch & Anna-Luisa Luger, Serine-dependent redox homeostasis regulates glioblastoma cell survival, British Journal of Cancer, 122, 1391–1398, 2020

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(Ort, Datum) (Unterschrift)