PHGDH Expression Is Required for Mitochondrial Redox Homeostasis, Breast Cancer Stem Cell Maintenance, and Lung Metastasis

Debangshu Samanta1,2, Youngrok Park1, Shaida A. Andrabi1,3, Laura M. Shelton4, Daniele M. Gilkes1,2,5, and Gregg L. Semenza1,2,5,6

Abstract

Intratumoral hypoxia stimulates enrichment of breast cancer stem cells (BCSC), which are critical for metastasis and patient mortality. Here we report a metabolic adaptation that is required for hypoxia-induced BCSC enrichment and metastasis. Hypoxia-inducible factors coordinately regulate expression of genes encoding phosphoglycerate dehydrogenase (PHGDH) and five downstream enzymes in the serine synthesis pathway and mitochondrial one-carbon (folate) cycle. RNAi-mediated silencing of PHGDH expression in both estrogen receptor-positive and negative breast cancer cells led to decreased NADPH levels, disturbed mitochondrial redox homeostasis, and increased apoptosis, which abrogated BCSC enrichment under hypoxic conditions. PHGDH-deficient cells exhibited increased oxidant levels and apoptosis, as well as loss of BCSC enrichment, in response to treatment with carboplatin or doxorubicin. PHGDH-deficient cells were relatively weakly tumorigenic and tumors that did form were deficient in BCSCs, abolishing metastatic capacity. Our findings highlight a role for PHGDH in the formation of secondary (recurrent or metastatic) tumors, with potential implications for therapeutic targeting of advanced cancers. Cancer Res; 76(15): 4430–42. ©2016 AACR.

Introduction

Breast cancer mortality occurs in patients whose cancer cells metastasize to distant sites, such as the lungs, bones, and brain. Only a small percentage of the breast cancer cells in a primary tumor have self-renewal capacity, which is necessary to form a metastatic tumor, and are designated as breast cancer stem cells (BCSC) or tumor-initiating cells (1, 2). Compared with bulk breast cancer cells, BCSCs exhibit increased survival when treated with cytotoxic chemotherapy (3, 4), which actively induces the breast cancer stem cell (BCSC) or tumor-initiating cells (1, 2). Compared with bulk breast cancer cells, BCSCs exhibit increased survival when treated with cytotoxic chemotherapy (3, 4), which actively induces the BCSC phenotype (5–7). Intratumoral hypoxia is common in advanced breast cancers (8) and induces the metastatic (9) and BCSC (10) phenotypes through transcriptional activation of target genes by hypoxia-inducible factor 1 (HIF-1) and HIF-2 (11–15). Adaptation of mammalian cells to chronic hypoxia involves a HIF-1–dependent switch from oxidative to glycolytic metabolism, which is an adaptive response to, and ameliorates, increased mitochondrial reactive oxygen species (ROS) production that occurs due to decreased electron transport chain efficiency under hypoxic conditions (16–23).

Oncogenic transformation also activates pathways that generate ROS and place cancer cells at risk for apoptosis (24). Redox homeostasis is dependent on a balance between levels of oxidants and antioxidants. The latter are dependent upon the generation of NADPH, which is used to maintain glutathione, the principal cellular antioxidant, in a reduced form. Two glycolytic shunt pathways utilize glucose metabolites for NADPH generation: the pentose phosphate pathway (PPP) converts glucose-6-phosphate, whereas the serine synthesis pathway (SSP) converts 3-phosphoglycerate into serine via three reactions that are catalyzed by phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH). Serine is utilized as a substrate for one-carbon (folate cycle) metabolism (1CM), either in the cytosol or mitochondria. In the mitochondria (mito1CM), serine hydroxymethyl transferase 2 (SHMT2) catalyzes the reaction of serine and tetrahydrofolate (THF) to glycine and 5,10-methylene-THF (MTHF). MTHF dehydrogenase 2 (MTHFD2) catalyzes the reaction of MTHF and NADPH+ to formyl-MTHF and NADP+. Finally, MTHFD1L splits formyl-THF into THF and formate (Fig. 1A). The cytosolic (cyto1CM) reactions are catalyzed by SHMT1 and MTHFD1 (which performs reactions catalyzed by both MTHFD2 and MTHFD1).

PHGDH catalyzes the reaction that diverts 3-phosphoglycerate from the Embden–Meyerhof pathway (EMP) to the SSP. A short hairpin RNA (shRNA) screen revealed that transformed breast cells required PHGDH expression for tumor xenograft formation (25). PHGDH gene amplification was found in 6% of breast cancers and shRNA-mediated knockdown of PHGDH expression inhibited proliferation of breast cancer cells with PHGDH amplification (25, 26). PHGDH overexpression was observed in 70% of estrogen receptor negative (ER−) breast cancers (25), indicating.

1Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, Maryland. 2McKusick-Nathans Institute of Genetics Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland. 3Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland. 4Human Metabolome Technologies America, Inc., Boston, Massachusetts. 5Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland. 6Departments of Pediatrics, Medicine, Radiation Oncology, and Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland. Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Gregg L. Semenza, Johns Hopkins University School of Medicine, 733 N. Broadway, Suite 671, Baltimore, MD 21205. Fax: 443-287-5688; E-mail: gsenenza@jhmi.edu
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that a mechanism other than gene amplification must underlie PHGDH overexpression in most breast cancers. We hypothesized that increased expression of PHGDH, as well as other SSP and 1CM enzymes, is required to maintain redox homeostasis in hypoxic breast cancer cells, especially in BCSCs, which are particularly sensitive to ROS (27).

Figure 1. Hypoxia induces expression of mRNAs encoding SSP and mito1CM enzymes.

A, enzymatic reactions. Glucose-derived 3-phosphoglycerate is metabolized to glycine and NADP⁺ is reduced to NADPH through the activity of SSP (blue) and mito1CM (purple) enzymes. B, mRNA expression. Breast cancer cell lines were exposed to 20% or 1% O₂ for 24 hours and expression of mRNAs encoding SSP and 1CM enzymes were analyzed by RT-qPCR. The expression of each mRNA was quantified relative to 18S rRNA and then normalized to the result obtained from MDA-MB-231 (MDA-231) cells at 20% O₂ (mean ± SEM; n = 3). C, summary of mRNA expression data (columns) in breast cancer cell lines (rows). Red, significantly increased expression at 1% as compared to 20% O₂ (P < 0.001; Student t test); gray, no induction at 1% O₂.
Materials and Methods

For details, see Supplementary Materials and Methods.

Cell culture
MCF-7, MDA-MB-231, HCC-1954, SUM-149, and SUM-159 cells were cultured as described previously (6). BT-474, ZR75.1, and T47D cells were cultured in RPMI1640 with 10% FBS. The cell lines were obtained from Dr. Sara Sukumar (Johns Hopkins University, Baltimore, MD) in 2012. Cell authentication was performed by PCR analysis of short tandem repeats.

Lentivirus transduction
Vectors encoding shRNA targeting HIF-1α and HIF-2α, and generation of MDA-MB-231 and MCF-7 subclones, were described previously (13, 28). plKO.1-puro lentiviral vectors encoding shRNA targeting PHGDH (Supplementary Table S1) were purchased from Sigma-Aldrich. Lentiviruses were packaged and transduced cells were selected as described previously (28).

Reverse transcription and quantitative real-time PCR
Total RNA was extracted from cells and tumors using TRIzol (Invitrogen) and treated with DNase I (Ambion). cDNA synthesis was performed using the iScript cDNA Synthesis system (Bio-Rad) and treated with DNase I (Ambion). cDNA synthesis was performed using the iScript cDNA Synthesis system (Bio-Rad). qPCR was performed using human-specific primers (Supplementary Table S1) and iQ SYBR Green Supermix (Bio-Rad; ref. 28).

Immunoblot assays
Whole-cell lysates were prepared in modified RIPA buffer (14). Blots were probed with antibodies against HIF-1α, PHGDH, PSAT1, and PSPH (Novus Biologicals). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotechnology) were used. Blots were reprobed with anti-actin antibody (Santa Cruz Biotechnology).

BCSC assays
Aldelhauer and mammosphere assays were performed as described previously (13).

MitoSoX staining
Cells were incubated in 5 μmol/L MitoSOX Red (Molecular Probes) in PBS/5% FBS at 37°C for 45 minutes and rinsed with PBS. Stained cells were filtered and subjected to flow cytometry.

Apopotosis and viability assays
Apopototic cells were quantified by FITC-Annexin V and APC-7-AAD staining followed by flow cytometry. Viable cells were quantified by MITT assay (Invitrogen).

Glutathione and NADPH assays
Cell lysates were analyzed for glutathione and NADPH using GSH/GSSG-Glo and NADP/NADPH-Glo assays (Promega).

Glucose uptake assay
Cells were incubated in 150 μmol/L 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)- amino]-2-deoxy-o-glucose (Molecular Probes) and subjected to flow cytometry.

Seahorse assays
Oxygen consumption and extracellular acidification were measured using the XF24-Analyzer (Seahorse Bioscience).

Metabolite analysis
Metabolites in culture media and cells were analyzed by capillary electrophoresis and single or tandem mass spectrometry (MS/MS) relative to internal standards (Human Metabolome Technologies) as described previously (29, 30).

Bioinformatics
For the HIF signature, the The Cancer Genome Atlas Breast Invasive Carcinoma Gene Expression Dataset of 1,215 patients was analyzed (31, 32). Tumor grade was analyzed using GOBO (33). Kaplan–Meier curves were generated using KM plotter (34).

Results

SSP and mito1CM enzyme expression is induced in hypoxic breast cancer cells
Breast cancers are classified as ER+, progesterone receptor positive (PR+), human epidermal growth factor receptor 2 positive (HER2+), or triple negative (ER-/PR-/HER2-). We exposed six representative human breast cancer cell lines to 20% or 1% O2 for 24 hours: BT-474 (ER-/PR+/HER2+), HCC-1954 (HER2+), MCF-7 (ER+/PR+/HER2-), MDA-MB-231 (ER+/PR-/HER2+), SUM-149 (ER-/PR-/HER2-), and ZR-75.1 (ER+, ref. 35). Total RNA was isolated and analyzed for expression of SSP (PHGDH, PSAT1, PSPH), mito1CM (SHMT2, MTHFD2, MTHFD1L), and cyto1CM (SHMT1, MTHFD1) mRNAs. In MDA-MB-231, HCC-1954, MCF-7 and BT-474 cells, hypoxic exposure induced the expression of all three SSP and all three mito1CM mRNAs, and all six breast cancer cell lines exhibited induction of PHGDH and SHMT2 mRNA (Fig. 1B and C), which encode enzymes catalyzing the first reaction of the SSP and mito1CM, respectively (Fig. 1A). In contrast, expression of cyto1CM mRNAs was induced by hypoxia in only one or two cell lines (Fig. 1C).

The SSP and PPP represent alternate mechanisms by which glucose metabolites are utilized to generate NADPH. The first enzyme of the PPP is glucose-6-phosphate dehydrogenase (G6PD). In contrast to the SSP and 1CM mRNAs, expression of G6PD mRNA was repressed by hypoxia in all breast cancer lines analyzed (Supplementary Fig. S1). Taken together, these data indicate that hypoxia selectively induces the expression of mRNAs encoding SSP and mito1CM enzymes in cell lines derived from ER+, PR+, HER2+, and triple-negative breast cancers.

HIFs are required for hypoxic induction of SSP and mito1CM enzymes
MDA-MB-231 subclones that were stably transfected with a vector encoding a nontargeting control shRNA (NTC) or shRNA targeting HIF-1α (sh1α), HIF-2α (sh2α), or both HIF-1α and HIF-2α (double knockdown (DDK)) have been used to investigate the role of HIFs in breast cancer progression (6, 13, 28). Hypoxic induction of PHGDH, PSAT1, PSPH, SHMT2, MTHFD2, and MTHFD1L mRNA expression, which was observed in the NTC subclone, was impaired when HIF-1α or HIF-2α or both were silenced (Fig. 2A). Immunoblot assays demonstrated hypoxic induction of PHGDH, PSAT1, and PSPH protein expression in the NTC subclone, which was impaired in the knockdown subclones (Fig. 2B). Similar results were obtained in MCF-7 subclones (Supplementary Fig. S2A). Hypoxia-induced PHGDH, PSAT1, and PSPH expression in parental MCF-7 cells was abrogated, in a dose-dependent manner, by treatment with acriflavine (Supplementary Fig. S2B), which is a drug that inhibits the
Figure 2.
SSP and mito1CM expression is HIF-dependent and increased in BCSCs. A, analysis of mRNA expression in MDA-MB-231 subclones, which expressed a non-targeting control shRNA (NTC) or shRNA targeting HIF-1α (sh1α), HIF-2α (sh2α), or both HIF-1α and HIF-2α (DKD), and were exposed to 20% or 1% O2 for 24 hours. Data were normalized to NTC at 20% O2 (mean ± SEM; n = 3). *, P < 0.01 versus NTC at 20% O2; #, P < 0.001 versus NTC at 1% O2.
B, immunoblot assays of lysates prepared from MDA-MB-231 subclones, which were exposed to 20% or 1% O2 for 48 hours. C, Aldefluor assay of MDA-MB-231 subclones exposed to 20% or 1% O2 for 72 hours. The percentage of cells expressing aldehyde dehydrogenase (ALDH+) was determined (mean ± SEM; n = 3). *, P < 0.05 versus NTC at 20% O2; #, P < 0.01 versus NTC at 1% O2. D, analysis of gene expression in adherent monolayers and mammospheres. MDA-MB-231 cells were cultured on standard or ultra-low adherence plates for 7 days in 20% O2 and adherent cells and mammospheres, respectively, were harvested for RT-qPCR analyses. Results were normalized to adherent cells (mean ± SEM; n = 3). *, P < 0.001 versus adherent cells (Student t test).
heterodimerization of HIF-α and HIF-β subunits (36). Thus, genetic and pharmacologic approaches indicate that HIFs coordinate to regulate the expression of SSP and mito1CM enzymes when breast cancer cells are exposed to hypoxia.

BCSCs are characterized by high aldehyde dehydrogenase (ALDH) activity and can be identified by the Aldefluor assay, in which BODIPY-aminoacetaldehyde is converted to the fluorescent product BODIPY-aminoacetate (37). Exposure of NTC subclones of MDA-MB-231 (Fig. 2C) and MCF-7 (Supplementary Fig. S2C) to hypoxia for 72 hours increased the percentage of ALDH+ BCSCs, whereas this response was impaired in knockdown subclones. Treatment of MCF-7 cells with acri-flavine also blocked hypoxic induction of the BCSC phenotype as determined by the mammosphere assay (Supplementary Fig. S2D), which is based on the selective ability of BCSCs to generate multicellular spheroids under nonadherent culture conditions (38).

Expression of SSP and mito1CM mRNAs is increased in BCSCs

The preceding results demonstrated a correlation between loss of hypoxia-induced SSP and mito1CM expression and loss of hypoxia-induced BCSC enrichment. To determine whether mRNAs encoding these enzymes were overexpressed in BCSCs relative to non-BCSCs, we cultured MDA-MB-231 and MCF-7 cells as either adherent monolayers or mammospheres for 7 days. HIF1α, HIF-2α, PHGDH, PSAT1, SHMT2, MTHFD2, and MTHFD1L mRNA expression was increased in BCSC-enriched mammosphere cultures of MDA-MB-231 cells, whereas expression of the cyto1CM enzymes SHMT1 and MTHFD2 was decreased in mammospheres relative to adherent cells, as was the PPP enzyme G6PD (Fig. 2D). Increased expression of HIF-1α, HIF-2α, PHGDH, SHMT2, MTHFD2, and MTHFD1L mRNA in BCSCs relative to non-BCSCs was also observed in MCF-7 cells (Supplementary Fig. S2E). Thus, HIF, SSP, and mito1CM mRNAs are preferentially expressed in BCSCs, suggesting that they play an important role in the BCSC phenotype.

PHGDH knockdown abrogates hypoxia-induced BCSC enrichment

We chose to analyze the effect of PHGDH loss-of-function in breast cancer cells for three reasons: (i) PHGDH is required for the diversion of glucose metabolites to the SSP and 1CM; (ii) PHGDH expression was hypoxia-inducible in all breast cancer lines analyzed; and (iii) PHGDH was preferentially expressed in BCSCs. MDA-MB-231 and MCF-7 cells were stably transfected with an shRNA targeting PHGDH (designated sh2 and sh4). Knockdown efficiency was validated at the mRNA (Fig. 3A) and protein (Fig. 3B) levels in both cell lines. PHGDH knockdown did not impair proliferation of either MDA-MB-231 (Supplementary Fig. S3A) or MCF-7 (Supplementary Fig. S3B) cells cultured for 72 hours at either 20% or 1% O2. In contrast, PHGDH knockdown markedly impaired the hypoxia-induced enrichment of BCSCs as determined by Aldefluor assays (Fig. 3C) or primary and secondary mammosphere assays (Fig. 3D). These results indicate that PHGDH expression is specifically required for hypoxic induction of the BCSC phenotype.

PHGDH is required to maintain redox homeostasis and survival of hypoxic breast cancer cells

Acute hypoxia leads to increased mitochondrial ROS generation (23). We hypothesized that PHGDH deficiency would lead to increased ROS levels and increased apoptosis. To test this hypothesis, we exposed MDA-MB-231 and MCF-7 subclones to 20% or 1% O2 for 72 hours in adherent culture and stained the cells with Mitosox Red, which is selectively targeted to mitochondria and generates fluorescence when oxidized by superoxide radicals, thereby serving as an indicator of mitochondrial ROS in live cells. The NTC subclones showed no increase in ROS after hypoxic exposure, whereas the percentage of Mitosox+ cells was significantly increased in PHGDH knockdown subclones (Fig. 3A). Analysis of Annexin V and 7-amino-actinomycin D (7-AAD) staining revealed no increase in apoptosis of hypoxic NTC cells, whereas the percentage of Annexin V+/7-AAD+ cells was increased in the PHGDH knockdown subclones (Fig. 3B). Exposure of cells to hypoxia in the presence of manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP), a cell-permeable superoxide scavenger (39), rescued the apoptosis of PHGDH knockdown subclones under hypoxia (Fig. 3C), indicating that increased apoptosis was due to increased ROS levels.

We hypothesized that PHGDH was required under hypoxic conditions for NADPH generation to maintain glutathione in a reduced state. Exposure of NTC subclones to hypoxia increased the ratio of reduced to oxidized glutathione (Fig. 3D), which was associated with a modest decrease in NADPH levels (Fig. 3E). In contrast, PHGDH knockdown was associated with an impaired hypoxic induction of reduced glutathione (Fig. 3D) and a significant decrease in NADPH levels (Fig. 3E). Thus, PHGDH deficiency impairs NADPH production, which becomes a liability specifically under hypoxic conditions.

PHGDH plays a major role in determining the utilization of glucose metabolites

Glucose metabolism via the EMP leads to the production of acetyl CoA, which is utilized for ATP generation through oxidative phosphorylation, and lactic acid, which is the terminal product of glycolysis. PHGDH diverts glucose metabolites to the SSP, thereby reducing production of both acetyl CoA and lactic acid. We analyzed the O2 consumption rate (OCR) and extracellular acidification rate (ECAR) to monitor oxidative phosphorylation and glycolysis, respectively, in NTC and PHGDH knockdown subclones. PHGDH deficiency increased the OCR (Fig. 4A) and ECAR (Fig. 4B) in both MDA-MB-231 and MCF-7 cells. Glucose uptake was not increased in PHGDH knockdown subclones (Fig. 4C). Taken together, these results indicate that the increased OCR and ECAR in PHGDH knockdown subclones are due to decreased shunting of glucose metabolites from the EMP to the SSP.

The effect of PHGDH knockdown on metabolite levels in MDA-MB-231 cells was analyzed by MS. PHGDH knockdown was associated with increased extracellular (Fig. 5D) and intracellular (Fig. 5F) lactic acid levels under both nonhypoxic and hypoxic conditions, which was consistent with the increased ECAR (Fig. 5B). Hypoxia increased extracellular serine levels in the NTC subclone, whereas PHGDH deficiency reduced serine levels under both nonhypoxic and hypoxic conditions (Fig. 5E). Thus, serine synthesis increases in NTC cells under hypoxic conditions as a result of increased PHGDH expression, leading to decreased import and/or increased export of serine. Hypoxia or PHGDH knockdown increased intracellular levels of all of the metabolites in the EMP at or downstream of the SSP branch point: 3-phosphoglyceric acid (Fig. 5G), 2-phosphoglyceric acid (Fig. 5H), phosphoenolpyruvic acid (Fig. 5I), and pyruvic acid (Fig. 5J). Hypoxia decreased intracellular levels of 6-phosphogluconic acid (Fig. 5K), the G6PD reaction product, which is consistent with decreased G6PD
expression in hypoxic cells (Supplementary Fig. S1). In contrast, PHGDH knockdown did not affect 6-phosphogluconic acid levels, as expected, because the PPP shunt is upstream of the SSP shunt in the EMP (Fig. 5L). Taken together, the data presented in Fig. 5 indicate that PHGDH shunts a significant proportion of glucose-derived 3-phosphoglycerate from the EMP to the SSP in breast cancer cells.

PHGDH knockdown increases the sensitivity of breast cancer cells to chemotherapy

As many cytotoxic cancer chemotherapies increase ROS levels (40), we hypothesized that PHGDH deficiency would also impair the enrichment of BCSCs that occurs in response to chemotherapy (5–7). Carboplatin is a chemotherapy agent that is used to treat breast cancer (41, 42). Platinum compounds increase mitochondrial ROS by forming adducts on mitochondrial DNA, thereby impairing the transcription of electron transport chain components (43). MDA-MB-231 and MCF-7 subclones were exposed to increasing concentrations of carboplatin for 72 hours and cell viability was determined by MTT assay. PHGDH deficiency sensitized breast cancer cells to carboplatin (Supplementary Fig. S4). Compared with NTC subclones, treatment of PHGDH-deficient cells with carboplatin at IC_{50} led to increased mitochondrial ROS (Fig. 6A) and apoptosis (Fig. 6B). Carboplatin treatment of NTC subclones induced enrichment of ALDH^{+} BCSCs, which was abrogated in the knockdown subclones (Fig. 6C).
Effect of PHGDH knockdown on redox homeostasis and cell survival. 

**Figure 4.** A, analysis of mitochondrial ROS production. MDA-MB-231 (left) and MCF-7 (right) subclones were exposed to 20% or 1% O2 for 72 hours and the percentage of cells positive for MitoSOX Red fluorescence was determined by flow cytometry (mean ± SEM; n = 3). #, P < 0.001 versus NTC at 1% O2.

B, analysis of apoptosis. The subclones were exposed to 20% or 1% O2 for 72 hours and the percentage of Annexin V+/7-AAD- apoptotic cells was determined (mean ± SEM; n = 3). **, P < 0.001 versus NTC at 20% O2; #, P < 0.001 versus NTC at 1% O2.

C, rescue by ROS scavenger. The subclones were exposed to 20% or 1% O2 in the presence or absence of 50 μmol/L MnTMPyP for 72 hours and the percentage of Annexin V+/7-AAD- apoptotic cells was determined (mean ± SEM; n = 3). ##, P < 0.001 versus NTC at 20% O2; #, P < 0.001 versus no MnTMPyP.

D and E, subclones were exposed to 20% or 1% O2 for 72 hours and the GSH/GSSG ratio (D) and NADPH levels (E) were measured and normalized to NTC at 20% O2 (mean ± SEM; n = 3). *, P < 0.01 versus NTC at 20% O2; #, P < 0.001 versus NTC at 1% O2.
Figure 5.
Metabolic consequences of PHGDH knockdown. A and B, measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). OCR (A; mean ± SEM; n = 3) and ECAR (B; mean ± SEM; n = 3) were measured in MCF-7 and MDA-MB-231 subclones incubated at 20% O2 for 72 hours. *P < 0.001 versus MCF-7 NTC; **P < 0.001 versus MDA-MB-231 NTC. C, analysis of glucose uptake. Subclones cultured under 20% O2 were stained with 150 μmol/L 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose and the mean fluorescence intensity was determined by flow cytometry (mean ± SEM; n = 3). D–K, metabolomic data. MDA-MB-231 subclones were exposed to 20% or 1% O2 for 72 hours and the absolute concentrations of extracellular (D and E) and intracellular (F–K) metabolites were determined by MS using reference standards (mean ± SEM; n = 3). *P < 0.001 versus NTC at 20% O2; †P < 0.001 versus NTC at 1% O2. L, glucose (Glc) metabolic pathways. Embden–Meyerhof (main) pathway, pentose phosphate pathway (PPP; only first reaction is shown), and serine synthesis pathway (SSP) are shown in abbreviated form that omits the five enzymatic reactions between glucose-6-phosphate (G6P) and 3PG, and the three reactions between 3PG and Ser.
Figure 6. Effect of PHGDH knockdown on the response to chemotherapy. A–C, response to chemotherapy in vitro. MDA-MB-231 (left) and MCF-7 (right) subclones were exposed to vehicle or carboplatin for 72 hours at \( \text{IC}_{50} \) (75 \( \mu \text{mol/L} \) for MDA-MB-231 and 200 \( \mu \text{mol/L} \) for MCF-7) and the percentage of MitoSOX\(^+\) (A), apoptotic (B), and ALDH\(^+\) (C) cells was determined (mean \pm SEM; \( n = 3 \)). *, \( P < 0.01 \) versus NTC at 20% O\(_2\); #, \( P < 0.001 \) vs. NTC at 1% O\(_2\).

D–G, response to chemotherapy in vivo. MDA-MB-231 subclones were implanted into the mammary fat pad of female NSG mice. When tumor volume reached 200 mm\(^3\) (day 0), the mice were randomly assigned to receive intraperitoneal injections of saline (Vehicle) or carboplatin (15 mg/kg) on days 0, 5, and 10. Tumors were harvested on day 13, and samples were analyzed for the percentage of MitoSOX\(^+\) (D), apoptotic (E), and ALDH\(^+\) (F) cells and the number of mammosphere-forming cells (G). Data are presented as mean \pm SEM (\( n = 3 \)). *, \( P < 0.01 \); **, \( P < 0.05 \), versus vehicle-treated NTC; #, \( P < 0.01 \) versus carboplatin-treated NTC.
We also investigated the effect of PHGDH knockdown on the response to doxorubicin, which is an anthracycline that is commonly used in the United States to treat breast cancer and was the first chemotherapeutic shown to induce HIF activity (44). Compared with NTC subclones, treatment of PHGDH-deficient cells with doxorubicin at IC_{50} increased mitochondrial ROS (Supplementary Fig. S5A) and apoptosis (Supplementary Fig. S5B). Doxorubicin treatment induced enrichment of ALDH{sup+} BCSCs, which was abrogated in the PHGDH-knockdown subclones (Supplementary Fig. S5C). Thus, both platino- and anthracycline chemotherapy induce PHGDH-dependent BCSC enrichment.

To investigate whether carboplatin-induced BCSC enrichment in vivo also requires PHGDH expression, MDA-MB-231 subclones (2 × 10^6 cells) were implanted in the mammary fat pad (MFP) of female NSG mice. When tumors reached a volume of 200 mm^3, mice were treated with 15 mg/kg of carboplatin by intraperitoneal injection every 5 days for 3 doses. Tumors were harvested 3 days after the last dose for analysis. Compared with NTC subclones, PHGDH knockdown subclones exhibited a greater increase in the percentage of MitoSOX{sup+} (Fig. 6D) and apoptotic (Fig. 6E) cells. Carboplatin treatment increased the percentage of ALDH{sup+} cells (Fig. 6F) and number of mammosphere-forming cells (Fig. 6G) in the NTC subclone, and these effects were abrogated by PHGDH knockdown (Fig. 6F and G). These results indicate that PHGDH deficiency sensitizes breast cancer cells to chemotherapy and abrogates chemotherapy-induced BCSC enrichment.

PHGDH expression promotes tumor initiation and is required for breast cancer metastasis

To investigate whether PHGDH regulates other aspects of breast cancer progression, MDA-MB-231 NTC and PHGDH knockdown subclones were implanted in the MFP of NSG mice and the resulting tumors were harvested on day 35. PHGDH knockdown was associated with a significant increase in tumor mass after orthotopic transplantation of sh2 and sh4 cells (Fig. 7A), which was consistent with cell culture data demonstrating increased growth of the sh2 and sh4 (as well as sh3 and sh5) PHGDH-knockdown subclones in vitro (Supplementary Fig. S3A). The tumors analyzed in Fig. 7A were derived from the injection of 2 × 10^6 cells; under these conditions, BCSCs are not limiting for tumor formation. PHGDH knockdown was associated with an increased percentage of MitoSOX{sup+} cells (Fig. 7B) and increased BCSCs as determined by Aldefluor (Fig. 7C) and mammosphere (Fig. 7D) assays. IHC revealed PHGDH expression in perinecrotic (hypoxic) regions of NTC tumors but not in knockdown tumors (Fig. 7E).

To determine whether PHGDH is required for spontaneous metastasis from breast to lungs, MDA-MB-231 subclones (2 × 10^6 cells) were again injected into the MFP of female NSG mice. To take into account differences in primary tumor growth, tumors and lungs were isolated when primary tumors reached a volume of 1000 mm^3. Despite growing faster, PHGDH knockdown tumors did not generate lung metastases, whereas in mice bearing NTC tumors, metastatic cells occupied large areas of the lung parenchyma (Fig. 7F).

To investigate whether PHGDH promotes the tumor-initiating potential of breast cancer cells, we injected only 1 × 10^3 cells into the MFP of female SCID mice, so that BCSCs would be limiting for tumor formation. NTC cells formed tumors by day 71 after injection in 7 of 7 injected mice, whereas sh2 and sh4 cells each formed tumors in only 3 out of 7 mice (Fig. 7G). PHGDH mRNA levels in knockdown tumors were significantly less than in NTC tumors (Fig. 7H).

Analysis of SSP and 1CM expression in primary human breast cancers

To investigate the clinical relevance of our experimental findings, we mined gene expression databases. We first investigated whether expression of mRNA encoding SSP and mito1CM enzymes, either individually or in aggregate (designated S1C), was correlated with the HIF signature, which comprised expression of HIF-1α mRNA and 13 HIF target-gene mRNAs (PLD1, VEGFA, LOX, P4HA2, NDRG1, SLC2A1, ERO1L, ADM, LDHA, PGK1, ANGPTL4, SLC2A3, and CA9), in 1,215 breast cancer specimens (31, 32) using Pearson correlation test. For each of the 6 mRNAs encoding an SSP or mito1CM enzyme, expression was significantly correlated with the HIF signature (P < 0.0001 in each case; Fig. 7I). These results are consistent with the data obtained from breast cancer cell lines demonstrating that hypoxia-induced expression of these genes is HIF-dependent (Fig. 1 and 2). Analysis of the GOBO database (33) revealed that S1C gene expression increased significantly (P < 0.00001) with increasing tumor grade (Supplementary Fig. S6A). Analysis of PHGDH or S1C mRNA expression in > 3,500 human breast cancer specimens using KM Plotter (34) revealed that levels greater than the median were associated with decreased relapse-free survival (HR = 1.34 and P < 10^{-7} for PHGDH; HR = 1.74 and P < 10^{-16} for S1C; Fig. 7J). Greater-than-median expression of SHMT2 or MTHFD2, but not PSAT1 or PSPH, was also associated with a significant decrease in relapse-free survival (Supplementary Fig. S6B). Thus, expression of SSP and mito1CM mRNAs in primary breast cancers is HIF-regulated and predictive of patient mortality.

Discussion

Recent studies have attempted to determine the mechanisms and consequences of PHGDH enzyme expression in breast cancer, but these studies have focused on cell proliferation in vitro or primary tumor growth in vivo (25, 26). Here, we demonstrate that hypoxia induces expression of PHGDH and other SSP and mito1CM enzymes that is mediated by HIF-1 and HIF-2. This coordinate regulation of multiple genes provides a mechanism to increase flux through the pathway. The expression of at least four out of six genes encoding SSP/mito1CM enzymes was induced by hypoxia in each of the cell lines analyzed, which included representative lines derived from ER{sup+}, HER2{sup+}, and triple-negative breast cancers. PHGDH and SHMT2 expression was hypoxia-induced in all six breast cancer lines and was increased in BCSC-enriched cell populations. In addition, increased expression in primary breast cancers of mRNA encoding PHGDH, SHMT2, MTHFD2, or all six SSP/mito1CM enzymes was associated with HIF target-gene expression and an increased risk of patient mortality. Regulation of PHGDH, PSAT1, PSPH, SHMT1, and SHMT2 expression by the transcription factors NRF2 and ATF4 in lung cancer and MYC in liver cancer was recently reported (45, 46). Further studies are required to determine whether ATF4 or MYC regulates basal expression of these genes in breast cancer. We studied the consequences of PHGDH deficiency because of its role as the first enzyme in the SSP that is responsible for diverting glucose metabolites from the EMP and because the finding of PHGDH gene amplification in 6% of primary breast...
Figure 7. Effect of PHGDH knockdown on orthotopic tumor initiation and metastasis, and clinical correlates. 

A–F, analysis of primary tumors and metastases. MDA-MB-231 subclones (2 × 10^6 cells) were implanted in the MFP of female NSG mice. After 35 days, the tumors and lungs were harvested. Tumor mass was determined (A) and tumor tissue was dissociated for analysis of MitoSOX^+ (B), ALDH^+ (C), and mammosphere-forming (D) cells (mean ± SEM, n = 3). *, P < 0.01 versus NTC. Tumor tissue was sectioned for PHGDH IHC (E; scale bar, 200 μm; N, necrosis). Lung sections (2 × 2 mm) were stained with hematoxylin and eosin to identify metastasis in three mice (M1, M2, M3), each bearing 1,000-mm^3 tumors derived from NTC or PHGDH knockdown cells (F). G and H, analysis of tumor-initiating capacity. MDA-MB-231 subclones were implanted in the MFP of female SCID mice (1 × 10^3 cells per mouse, 7 mice per subclone). Mice were scored for palpable tumors after 71 days. *, P < 0.05 vs. NTC, c^2 test (G). RNA was extracted from those tumors that formed, PHGDH mRNA levels were determined, and results were normalized to NTC (mean ± SEM; n = 3); *, P < 0.01 versus NTC (H). I, analysis of gene expression data from human primary breast cancers. Pearson correlation test was performed to compare expression of the HIF signature with expression of the six SSP/mito1CM mRNAs in aggregate (S1C) or individually using data from 1,215 breast cancer samples. *, P < 0.0001, two-tailed t test. J, correlation of gene expression with patient mortality. Kaplan-Meier analyses of relapse-free survival over ten years were performed using data for 3,554 breast cancer patients, who were stratified by PHGDH (left) or S1C (right) mRNA expression levels in the primary tumor, which were greater (red) or less (black) than the median level. The hazard ratio (HR) and P value (log-rank test) for each comparison are shown.
cancers suggested a critical role for this enzyme in breast cancer progression. Our data suggest that a major determinant of increased PHGDH expression in breast cancers without gene amplification is intratumoral hypoxia, which is a common finding in advanced breast cancer (8). The correlation between expression of PHGDH and HIF target genes in primary breast cancers strongly supports this conclusion. Similar to PHGDH, SHMT2 expression was induced by hypoxia in all breast cancer lines studied and SHMT2 overexpression in breast cancers was associated with patient mortality, suggesting that SHMT2 is also important for breast cancer progression. The expression of SHMT2 is induced by hypoxia in neuroblastoma cells and SHMT2 knockdown was shown to increase ROS levels and cell death under hypoxic conditions, but effects on cancer stem cells and metastasis were not studied (47). These findings point to the importance of coordinate, HIF-mediated regulation of SSP and mito1CM enzyme expression, and suggest that this metabolic requirement is not limited to breast cancer.

Metabolic analyses revealed that both ER+ and ER− breast cancer cells divert a considerable proportion of glucose-derived 3-phosphoglyceric acid to the SSP, as PHGDH knockdown led to significantly increased oxidative and glycolytic metabolism under nonhypoxic conditions. Consistent with these findings, metabolic analyses revealed that PHGDH knockdown led to increased concentrations of EMP intermediates downstream of and including 3-phosphoglyceric acid, the substrate for PHGDH. Gene expression and clinical outcome data from thousands of breast cancers suggest that this metabolic reprogramming is not limited to tissue culture cells. The orthotopic transplantation studies provide evidence that PHGDH expression is a major determinant of BCSC abundance and metastasis.

Our data indicate that PHGDH expression is critical under hypoxic conditions to increase the availability of reduced glutathione for maintenance of redox homeostasis. In PHGDH knockdown subclones, NADPH levels were decreased and mitochondrial ROS levels increased under hypoxic conditions, leading to increased apoptosis compared with NTC subclones. Gene expression and metabolic studies revealed increased expression of the first enzyme of the SSP and mito1CM (PHGDH and SHMT2) and repression of the first enzyme of the PPP (G6PD) in hypoxic breast cancer cells, indicating a switch from cytosolic to mitochondrial production of NADPH to combat increased ROS generated by the electron transport chain (23). Thus, under hypoxic conditions, HIFs mediate a metabolic switch from oxidative to glycolytic metabolism, which reduces mitochondrial oxidant generation (18), and a switch from cytosolic to mitochondrial NADPH generation, which augments antioxidant defenses. The increase in NADPH complements increased glutathione synthesis, which is also induced in hypoxic breast cancer cells by HIF−1 (7, 48).

PHGDH knockdown reduced the number of BCSCs under both nonhypoxic and hypoxic conditions in vitro and within orthotopic tumors. The functional consequences of this loss of BCSCs included impaired capacity for tumor initiation and lung metastasis. We interpret the previously reported failure of PHGDH knockdown cells to form tumor xenografts (25) as due to a deficiency of BCSCs rather than an effect on cell proliferation, which we did not observe in vitro or in vivo. Similarly, the loss of metastatic capacity we demonstrated is likely due, at least in part, to a deficiency of BCSCs (49, 50).

PHGDH knockdown sensitized both ER+ and ER− breast cancer lines to chemotherapy, with increased mitochondrial ROS, increased apoptosis, and loss of chemotherapy-induced BCSC enrichment. These findings suggest that combining chemotherapy with an inhibitor of PHGDH may improve the survival of women with advanced breast cancer by blocking counter-therapeutic induction of BCSCs. HIF inhibitors may be useful to target BCSCs because, in addition to blocking induction of SSP and mito1CM enzymes, they block multiple other pathways, which are induced by hypoxia (13, 14) or chemotherapy (6, 7) and which promote BCSC specification or maintenance. Our findings underscore the importance of adaptive responses to the hypoxic tumor microenvironment and to chemotherapy, whereby cancer cells maintain metabolic and redox homeostasis, which is required for execution of the BCSC and metastatic programs that underlie the lethal cancer phenotype.

Disclosure of Potential Conflicts of Interest
The authors disclose no potential conflicts of interest.

Authors’ Contributions
Conception and design: D. Samanta, G.L. Semenza
Development of methodology: D. Samanta, S.A. Andrabi, G.L. Semenza
Acquisition of data (provided animals, collected samples, provided facilities): D. Samanta, Y. Park, S.A. Andrabi, D.M. Gilles
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Samanta, S.A. Andrabi, G.L. Semenza
Writing, review, and/or revision of the manuscript (i.e., reporting or organizing data, constructing databases): J.M. Shelton, G.L. Semenza
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Shelton
Study supervision: G.L. Semenza

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Debangshu Samanta, Youngrok Park, Shaida A. Andrabi, et al.

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