

Serine Synthesis Helps Hypoxic Cancer Stem Cells Regulate Redox

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Abstract

Phosphoglycerate dehydrogenase (PHGDH) is the metabolic enzyme responsible for shunting the glycolytic intermediate 3-phosphoglycerate to the serine synthesis pathway. In breast cancer and several other types of cancer, increased PHGDH expression is associated with patient mortality. Early studies focused on the role of PHGDH in promoting cell proliferation in the small percentage of breast cancers with *PHGDH* gene amplification. However, recent studies have revealed a critical role for PHGDH

and downstream enzymes of the serine synthesis pathway and one carbon metabolism in NADPH production and the maintenance of redox homeostasis, which are required for enrichment of breast cancer stem cells in response to hypoxia or chemotherapy. These results provide a mechanism for PHGDH overexpression in breast cancers in which PHGDH is not amplified and have implications for improving the response of triple-negative breast cancers to cytotoxic chemotherapy. *Cancer Res*; 76(22); 6458–62. ©2016 AACR.

Intratumoral Hypoxia Is a Critical Driving Force for Cancer Progression

Within tumors, O₂ levels rapidly decline as distance from the nearest blood vessel increases, such that a fundamental basis for cancer cell heterogeneity is O₂ availability, with one quarter of breast cancer tissue having a PO₂ of 2.5 mm Hg (0.4% O₂) or less (1). Median intratumoral PO₂ <10 mm Hg (1.4% O₂) is associated with decreased disease-free survival in studies involving >700 patients with cervical cancer and >500 patients with head and neck cancers (1). Reduced intracellular O₂ concentrations induce the expression of hypoxia-inducible factors (HIF), which are transcriptional activators composed of an O₂-regulated HIF- α subunit (HIF-1 α , HIF-2 α , or HIF-3 α) and a constitutively expressed HIF-1 β subunit (2, 3). The HIF- α subunits are subjected to O₂-dependent prolyl hydroxylation, ubiquitination, and proteasomal degradation, which are inhibited under hypoxic conditions (3), leading to stabilization and rapid accumulation of the proteins and transcriptional activity.

Intratumoral hypoxia is the single most important feature of the microenvironment driving cancer progression. HIFs activate the transcription of a large battery of genes encoding proteins that control every step of the metastatic process, including vascularization, stromal cell recruitment, extracellular matrix remodeling, premetastatic niche formation, cell motility, local tissue invasion,

intravasation, and extravasation at sites of metastasis (4). In addition, hypoxia increases the percentage of breast cancer stem cells (BCSC; ref. 5), which are a small subset of cells within the primary tumor that have the capacity to generate secondary tumors (4), in an HIF-dependent manner.

Hypoxia Increases Mitochondrial Production of Reactive Oxygen Species

Malignant transformation induces reprogramming of cell metabolism to support tumor growth, tissue remodeling, and cancer metastasis. This switch is regulated by oncogenes and tumor suppressor genes and is influenced by the tumor microenvironment. All human cells require a constant supply of O₂ to carry out oxidative phosphorylation in the mitochondria for ATP generation. O₂ is utilized as the final electron acceptor in the mitochondrial electron transport chain (ETC), resulting in the generation of water (Fig. 1). Under hypoxic conditions, ETC efficiency is impaired, resulting in the reaction of electrons with O₂ to form superoxide radicals rather than water, which causes cell death if it is not regulated (6). Mitochondrial reactive oxygen species (ROS) also induce HIF activity (6), which mediates adaptation to hypoxia in two ways: (i) cell proliferation is inhibited to prevent any further increase in the number of O₂-consuming cells; and (ii) metabolism is reprogrammed to maintain cellular redox homeostasis (7, 8). In this review, we will discuss the role of HIFs in regulating cellular metabolism and redox status, which enables enrichment of the BCSC population under hypoxic conditions.

Cells Adapt to Hypoxia by Shifting from Oxidative to Glycolytic Metabolism

Reprogramming of cellular metabolism toward increased glycolysis and suppressed oxidative phosphorylation is a major adaptation to hypoxia. Although this metabolic switch was previously interpreted as a means of maintaining energy production, studies of HIF-1 α -deficient mouse embryo fibroblasts, which did not switch from oxidative to glycolytic metabolism when ambient

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doi: 10.1158/0008-5472.CAN-16-1730

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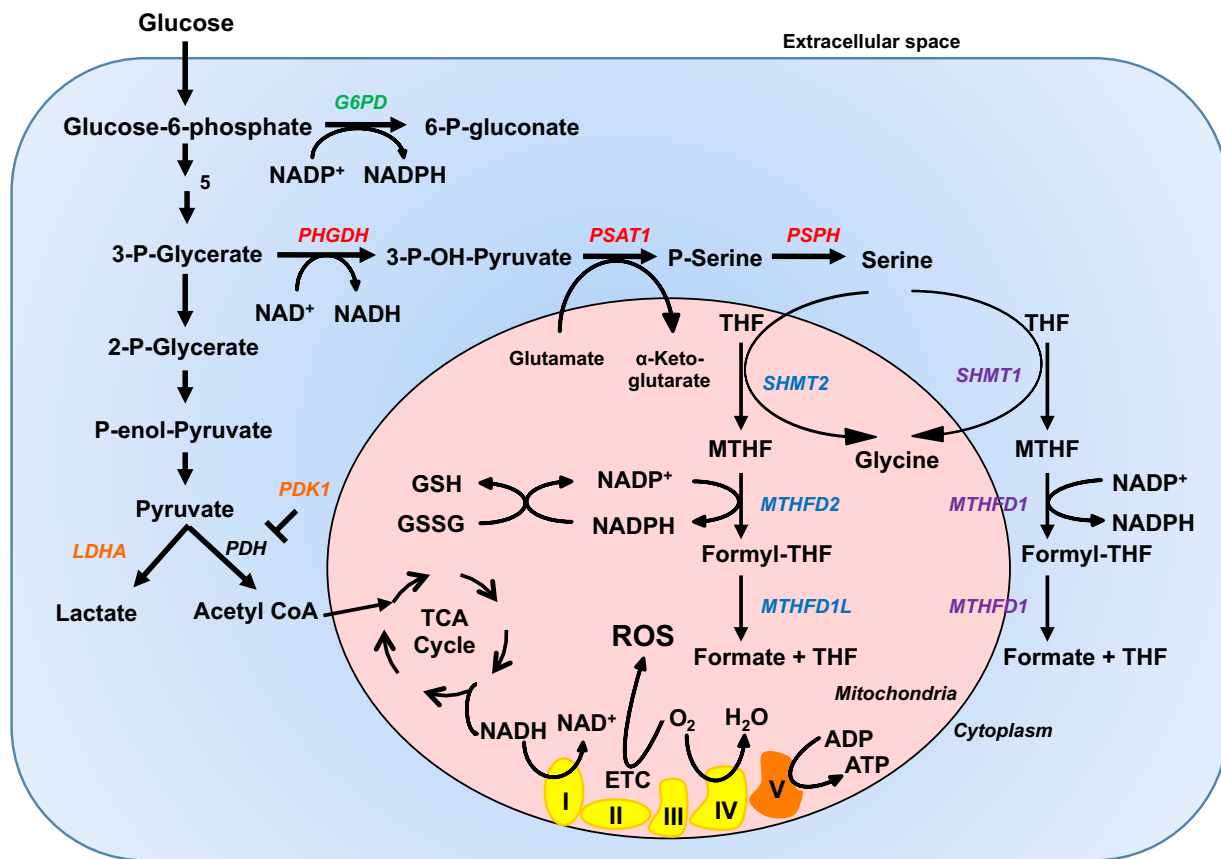


Figure 1.

Regulation of the Embden–Meyerhoff and shunt pathways in breast cancer cells. Under hypoxic conditions, mitochondrial generation of ROS by the ETC increases. In response, HIFs activate transcription of *LDHA* and *PDK1* (orange) to decrease the generation of acetyl CoA for entry into the tricarboxylic acid (TCA) cycle; *PHGDH*, *PSAT1*, and *PSPH* (red) to increase conversion of glucose to serine (serine synthesis pathway); and *SHMT2*, *MTHFD2*, and *MTHFD1L* (blue) to increase generation of mitochondrial NADPH (mitochondrial one-carbon metabolism), which is required to convert glutathione from oxidized (GSSG) to reduced (GSH) form. Genes required for generation of cytosolic NADPH (cytosolic one-carbon metabolism) are either not consistently induced (*SHMT1*, *MTHFD1*, purple) or actively repressed (*G6PD*, green) under hypoxic conditions. *LDHA*, lactate dehydrogenase A; *PDK1*, pyruvate dehydrogenase kinase 1; *SHMT2*, serine hydroxymethyltransferase 2; *MTHFD2*, methylene tetrahydrofolate dehydrogenase 2; *MTHFD1L*, methylene tetrahydrofolate dehydrogenase 1-like; *SHMT1*, serine hydroxymethyltransferase 1; *MTHFD1*, methylene tetrahydrofolate dehydrogenase 1.

O₂ levels were decreased from 20% O₂ (140 mm Hg) to 1% O₂ (7 mm Hg), revealed that ATP production was not compromised but the cells died due to excess levels of ROS (9, 10). HIF-1-dependent expression of pyruvate dehydrogenase kinase 1 and lactate dehydrogenase A suppresses oxidation of pyruvate to acetyl CoA and increases the reduction of pyruvate to lactate, respectively (9, 11–13). HIF-1 represses expression of medium and long-chain acyl-CoA dehydrogenases, thereby suppressing fatty acid oxidation (14). Finally, HIF-1-dependent expression of BNIP3 stimulates mitochondrial autophagy, which suppresses both glucose and fatty acid oxidation (10).

Glycolytic Shunt Pathways Produce NADPH

Cellular redox homeostasis represents a balance between oxidants (principally, ROS) and antioxidants (principally, reduced glutathione). Antioxidant defense is dependent on the generation of NADPH, which is used to maintain glutathione in a reduced form. Two different glycolytic shunt pathways generate NADPH: the pentose phosphate pathway (PPP) and the combined activity

of the serine synthesis pathway (SSP) and one-carbon (folate cycle) metabolism (1CM). In the PPP, glucose-6-phosphate dehydrogenase (*G6PD*) converts the glycolytic intermediate glucose-6-phosphate and NADP⁺ to 6-phosphogluconate and NADPH in the cytosol (Fig. 1). In the SSP, the glycolytic intermediate 3-phosphoglycerate is converted to serine via three reactions, which are catalyzed by phosphoglycerate dehydrogenase (*PHGDH*), phosphoserine aminotransferase 1 (*PSAT1*), and phosphoserine phosphatase (*PSPH*). Serine is then utilized for 1CM, either in the cytosol or mitochondria, which generates glycine and NADPH (Fig. 1).

PHGDH Is Overexpressed in Cancer Cells

PHGDH is overexpressed in the triple-negative subtype of breast cancer (TNBC), and *PHGDH* gene amplification was observed in 6% of breast cancers (15, 16). High-level *PHGDH* protein expression in both normal and neoplastic breast tissue was associated with a keratin 5-positive cell lineage, thereby establishing an association of this protein with the basal

phenotype (17), which is enriched for stem cells. A short hairpin RNA (shRNA) screen revealed that a transformed breast cell line required PHGDH expression for tumor xenograft formation (16). In breast and melanoma cancer cell lines, *PHGDH* gene amplification was associated with increased proliferation (15). shRNA-mediated PHGDH knockdown in MDA-MB-468, a TNBC cell line with *PHGDH* amplification, was reported to decrease xenograft growth (16), but this result was not replicated (18). PHGDH overexpression was also reported in non-small cell lung, cervical, and colorectal cancers (19–21). The *BRAF*^{V600E} mutation, which occurs in approximately 45% of papillary thyroid carcinomas, was correlated with higher PHGDH protein expression (22). Recently, small-molecule inhibitors of PHGDH were identified that decrease the production of glucose-derived serine and suppress the proliferation of PHGDH-dependent cancer cells in tissue culture (23, 24). Administration of an inhibitor to mice increased necrosis in MDA-MB-468 tumor xenografts but had no effect on xenografts derived from MDA-MB-231 cells, which do not have *PHGDH* gene amplification (24). Taken together, these studies suggested that PHGDH expression is increased in a variety of cancers and that PHGDH inhibition impaired proliferation of breast cancer cells with *PHGDH* gene amplification, which accounts for only a small percentage of breast cancers in which PHGDH is overexpressed.

Glycolytic Shunt Pathways Are Reprogrammed in Hypoxic Breast Cancer Cells

Breast cancers are classified according to expression of the estrogen receptor (ER), progesterone receptor (PR), and HER2. Analysis of ER⁺ (ZR75.1), ER⁺PR⁺ (MCF-7), ER⁺PR⁺HER2⁺ (BT-474), HER2⁺ (HCC-1954), and TNBC (MDA-MB-231, SUM-149) cell lines, none of which have *PHGDH* gene amplification, revealed that *PHGDH* expression was induced by hypoxia in an HIF-dependent manner in all cell lines; in contrast, *G6PD* expression was repressed by hypoxia in all six lines, indicating a reprogramming of glucose metabolism to increase flux through the SSP and decrease flux through the PPP under hypoxic conditions, which was confirmed by metabolomic analyses (25).

Knockdown of PHGDH expression (PHGDH-kd) by shRNA had no effect on the proliferation of MCF-7 cells and increased the proliferation of MDA-MB-231 cells (25), which stands in contrast to the results reported above for MDA-MB-468 cells. The most dramatic effects of PHGDH loss of function were observed under hypoxic conditions, which increased mitochondrial ROS levels and apoptosis in PHGDH-kd subclones of both cell lines, but not in subclones expressing a nontargeting control shRNA (NTC; ref. 25). The ratio of reduced-to-oxidized glutathione increased dramatically under hypoxic conditions in NTC but not in PHGDH-kd subclones, which also had significantly decreased levels of NADPH (25).

HIFs Coordinately Regulate Expression of SSP and Mitochondrial 1CM Enzymes

The major role of 1CM in NADPH generation and redox regulation has only recently become appreciated (26). Analysis of gene expression revealed that in addition to PHGDH, hypoxia induced the expression of the other two enzymes of the SSP (PSAT1 and PSPH) and all three enzymes required for mitochon-

drial 1CM (SHMT2, MTHFD2, and MTHFD1L) in an HIF-dependent manner, whereas the enzymes required for cytosolic 1CM (SHMT1, MTHFD1) were not hypoxia-induced in the majority of cell lines (25). These results suggest that HIF-dependent induction of the SSP and mito1CM represents another metabolic adaptation to hypoxia. However, in contrast to those metabolic switches described earlier in this review, which serve to decrease production of oxidants, this adaptation serves to increase the production of antioxidants.

It should be noted that although HIFs play a critical role in activating transcription of genes encoding SSP and mitochondrial 1CM enzymes in hypoxic breast cancer cells, other transcription factors are responsible for basal transcription of these genes. In lung cancer, nuclear factor erythroid 2 (NRF2) activates transcription of the *ATF4* gene that encodes activating transcription factor 4, which directly transactivates the *PHGDH*, *PSAT1*, and *SHMT2* genes (19).

As in the case of PHGDH (the first enzyme of the SSP), expression of SHMT2 (the first enzyme of mitochondrial 1CM) was induced by hypoxia in all breast cancer cell lines analyzed (25). Knockdown of SHMT2 in neuroblastoma cell lines increased oxidant stress and cell death under hypoxic conditions and, in clinical samples, *SHMT2* and *PHGDH* expression were more highly correlated in biopsies of neuroblastoma patients who died ($r = 0.90$) as compared with biopsies of survivors ($r = 0.42$; ref. 27), suggesting that a coupling of the SSP to mitochondrial 1CM was important for disease progression. SHMT2 expression promoted survival of glioblastoma cells in the hypoxic tumor microenvironment (28), but the connection to redox homeostasis was not investigated. These results suggest that knockdown of SHMT2, MTHFD2, or MTHFD1L may have a greater effect on redox homeostasis under hypoxic conditions than knockdown of SHMT1 or MTHFD1 (which performs the reactions catalyzed by both MTHFD2 and MTHFD1L), but further studies are required to test this hypothesis.

The SSP Plays a Critical Role in Regulating BCSCs and Metastasis

Only a small percentage of the cancer cells in a primary breast tumor have self-renewal capacity, which is necessary to form a metastasis or recurrent tumor, and these cells are designated as tumor-initiating cells or BCSCs (29). They are also resistant to chemotherapy. On the basis of prior studies of hematopoietic stem cells (30), we hypothesized that BCSCs were particularly sensitive to disturbance of redox homeostasis. HIF-1 α , HIF-2 α , PHGDH, SHMT2, MTHFD2, and MTHFD1L mRNAs were expressed at higher levels when MCF-7 or MDA-MB-231 cells were cultured under conditions that enriched for BCSCs, whereas *G6PD* mRNA was expressed at lower levels in BCSCs, and PHGDH knockdown markedly impaired the hypoxia-induced enrichment of BCSCs (25). These results indicated that PHGDH expression was specifically required for hypoxic induction of the BCSC phenotype, which was similar to the effect of HIF-1 α knockdown (5, 31).

When 2×10^6 MDA-MB-231 cells are implanted into the mammary fat pad of immunodeficient mice, BCSCs are not limiting for tumor growth, and both NTC and PHGDH-kd subclones gave rise to tumors in all mice. Remarkably, the growth of PHGDH-kd primary tumors was significantly increased, but the percentage of BCSCs in these tumors was decreased 2- to 4-fold.

When only 1×10^3 cells were implanted, BCSCs were limiting, and whereas tumors grew in 100% of the mice implanted with NTC subclones, less than 50% of mice injected with PHGDH-kd cells developed tumors within 10 weeks and, in the mice that formed primary tumors, lung metastasis was dramatically impaired (25).

Further evidence for an effect of PHGDH on pluripotency and self-renewal was provided by a recent study of mouse embryonic stem cells in which PHGDH knockdown was associated with decreased expression of core pluripotent factors and impaired self-renewal (32). The finding that PHGDH expression is required for breast cancer metastasis (25) is consistent with a report that a highly bone metastatic subclone of MDA-MB-231 had increased PHGDH expression compared with the parental MDA-MB-231 cells (33). In glioma, inhibition of PHGDH expression reduced cell invasion *in vitro* (34). Both cytosolic and mitochondrial ROS levels were increased in metastatic melanoma nodules, as compared with subcutaneous tumors, and knockdown of MTHFD1, a cytosolic enzyme that generates NADPH, or ALDH1L2, a mitochondrial enzyme that generates NADPH, limited distant metastasis without affecting the growth of subcutaneous tumors (35). However, no experiments were performed to interrogate the role of cancer stem cells. Administration of the glutathione precursor N-acetyl cysteine to tumor-bearing mice increased the number of lymph node metastases but had no impact on the number or size of primary melanomas (35, 36).

Clinical Relevance

Whereas ER⁺/PR⁺ breast cancers are treated with tamoxifen or aromatase inhibitors and HER2⁺ cancers are treated with trastuzumab or tyrosine kinase inhibitors, there is no targeted therapy for TNBC. The administration of cytotoxic chemotherapy leads to remission, which is unfortunately followed by an increased incidence of cancer recurrence, metastasis, and patient mortality. Exposure of PHGDH-kd subclones to cytotoxic chemotherapy (carboplatin or doxorubicin) led to increased mitochondrial ROS and apoptosis. Furthermore, whereas treatment of NTC subclones

with chemotherapy led to a marked increase in the percentage of BCSCs among the surviving cell population, no enrichment of BCSCs was observed after treatment of the PHGDH-kd subclones (25). These results suggest that PHGDH inhibitors may improve the response to cytotoxic chemotherapy. Inhibition of PHGDH should impair both cytosolic and mitochondrial NADPH production. Alternatively, it is possible that inhibitors of mitochondrial 1CM enzymes may provide a greater therapeutic window, as the mRNAs encoding these enzymes show the greatest increase in cancer compared with normal tissue of any metabolic pathway (37). Another potential therapeutic approach is to target HIFs for inhibition (38). HIF inhibitors block chemotherapy-induced enrichment of BCSCs (39, 40) but also block additional downstream targets that play critical roles in the metastatic process (4). Finally, if oxidative stress limits distant metastasis, as mouse models of breast cancer and melanoma suggest, then treatment of cancer patients with antioxidants may be contraindicated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Samanta, G.L. Semenza
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Samanta, G.L. Semenza
Writing, review, and/or revision of the manuscript: D. Samanta, G.L. Semenza
Study supervision: G.L. Semenza

Grant Support

This work was supported by Research Professor Award 122437-RP-12-090-01-COUN from the American Cancer Society, Impact Award W81XWH-12-1-0464 from the Department of Defense Breast Cancer Research Program, and a grant from the Cindy Rosencrans Fund for Triple Negative Breast Cancer.

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Received June 22, 2016; revised August 31, 2016; accepted September 2, 2016; published OnlineFirst November 3, 2016.

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