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-1α, HIF-2α, or HIF-3α) and a constitutively expressed HIF-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-1α–deficient mouse embryo fibroblasts, which did not switch from oxidative to glycolytic metabolism when ambient...
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

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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.
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 BRAFV600E 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 their 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 been 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 PSH) and all three enzymes required for mitochondrial 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 mitochondrial 1CM 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 ATR3-4 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-1α, HIF-2α, 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-1α knockdown (5, 31).

When 2 × 10⁶ 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 $1 \times 10^3$ 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 but had no impact on the number or size of primary tumors (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 cell invasion (33). In glioma, inhibition of PHGDH expression reduced PHGDH expression compared with the parental MDA-MB-231 cell line (34). In breast cancer, the highly bone metastatic subclone of MDA-MB-231 had increased PHGDH expression (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).

References

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