



Redox regulation of hybrid metabolic state in breast cancer metastasis

Kuldeep S. Attri¹, Jun Hyoung Park¹, Benny Abraham Kaiparettu^{1,2}

¹Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA; ²Dan L. Duncan Comprehensive Cancer Center, Baylor College of Medicine, Houston, TX, USA

Correspondence to: Benny Abraham Kaiparettu. Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA. Email: kaipare@bcm.edu.

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Reactive oxygen species (ROS) homeostasis is critical for regulating bifunctionality in cellular responses. While low levels of ROS activate oncogenic signaling by redox mechanisms, high levels of ROS referred to as oxidative stress damages DNA, protein, and lipids (1). ROS can be generated either in the mitochondria or cytosol by different mechanisms (2). To circumvent the mounting ROS levels, cancer cells are equipped with multiple antioxidant enzyme systems. Surprisingly, redox systems can have contrasting outcomes in diverse cancer types, tumor microenvironments, different stages of cancer progression, and metastasis (3).

The recent study published in *Proceedings of the National Academy of Sciences* by Ren and colleagues addresses the increased oncogenic potential by the loss of an antioxidant gene, glutathione peroxidase 2 (GPx2) (4). Contrary to its oncogenic role in previous studies on multiple cancers (5-7), this study describing GPx2 as a tumor suppressor in breast cancer. Analysis of patient data suggested that higher expression of GPx2 is associated with better survival outcome in patients with luminal B, HER2-enriched and Basal-like breast cancer subtypes. Utilizing robust overexpression and knockdown in murine and human cancer cell models, the paper demonstrates the negative impact of GPx2 in both tumor growth and lung metastasis (4). These data underscore the context-dependent function of the GPx2-mediated redox mechanism in breast cancer.

Altered cellular metabolism and phenotypic plasticity are key hallmarks of cancer cells often associated with

angiogenesis, invasion, and metastasis (8). Though rapidly proliferating cancer cells usually rely on the Warburg effect or aerobic glycolysis to meet their increasing demands of energy and biomass production (9,10), recent studies suggest that circulating cancer cells that are involved in metastatic transformation, actively utilize mitochondrial oxidative phosphorylation (OXPHOS) for their bioenergetic needs (11). Lactate, a byproduct of aerobic glycolysis, apart from making the tumor microenvironment acidic, is utilized by the cancer cells to stabilize HIF-1 α in a positive feedback loop. mtROS also stabilizes HIF-1 α by inhibition of prolyl hydroxylase domain protein (PHD) to elicit a cellular response to hypoxia (12). HIF-1 α regulates tumor metabolism, angiogenesis, proliferation, stemness, and epithelial to mesenchymal transition (13). Cancer cells can also maintain high anaplerotic flux into the tricarboxylic acid (TCA) cycle to support OXPHOS by alternative metabolic sources including glutamine oxidation. Cancer cells rely on growth factor signaling to generate glycolytic or glutaminolytic fluxes to meet nutrient demands from the changing tumor microenvironment (10). Mitochondrial fatty acid oxidation (FAO) is also a major energy source for cancer subtypes like the triple negative breast cancer (14). However, the rapid proliferation of cancer cells generates a hypoxic tumor microenvironment. HIF-1 α signaling in a hypoxic microenvironment regulates aerobic glycolysis to promote tumor cell proliferation (13,15).

The metastatic transition of breast cancer cells displays phenotypic plasticity associated with distinct metabolic

pathways. Both hypoxia and ROS, are known modifiers of epithelial and metastatic states. While proliferative epithelial cells rely majorly on aerobic glycolysis, the quiescent mesenchymal cells depend on OXPHOS for metabolic needs (16). Using loss- and gain- of function studies in mice, Ren *et al.* demonstrate that GPx2-regulated ROS activates HIF-1 α signaling. Consequent to HIF-1 α activation, cancer cells show a metabolic shift towards aerobic glycolysis and increased vasculogenesis via VEGFA induction. Further, the rapid formation of defective vessels exacerbated hypoxia in tumors potentiating ROS-mediated stabilization of HIF-1 α . Although previous studies have shown the biphasic effect of ROS in tumorigenesis and metastasis, herein, both aerobic glycolysis and high ROS led to enhanced tumorigenesis, and spontaneous lung metastasis (4). Thus, this study provides substantial evidence for plasticity in the dichotomous regulation of ROS in cancer progression.

The advances in single-cell sequencing technology have unraveled the heterogeneity in the metabolic plasticity of cancer cells. Identification of gene regulatory networks has stratified tumor cells in distinct functional states associated with cellular phenotypes (17). Previous studies from our group have identified a metabolic hybrid cancer cell state by utilizing an integrated analysis of transcriptional regulatory networks, metabolic pathways, and mathematical modeling. Unlike the normal healthy cells that exclusively maintain either glycolytic or OXPHOS metabolic state depending on their oxygen availability, cancer cells exhibit an additional stable hybrid metabolic state with increased HIF-1 α stabilization and AMPK activity for elevated glycolysis and OXPHOS pathways. The hybrid metabolic state was further identified in multiple cancer patient's data from TCGA, and single-cell sequencing data of cancer cells based on HIF-1 α and AMPK transcriptomic gene signatures (18-20). In general, the metabolic switch between the glycolytic or oxidative metabolic states is mediated by reciprocal regulation of HIF-1 α and AMPK-regulated proteins that senses different cellular stresses (21). HIF-1 α is an oxygen sensor that increases ROS levels by positive feedback regulation (15). While the energy sensor AMPK dampens the levels of ROS by regulating antioxidant genes, AMPK also induce ROS generation by activating mitochondrial biogenesis and electron transport chain, underscoring the significance of AMPK in dictating ROS signaling and metabolic fates (22,23). Thus, the interplay of HIF-1 α stabilization, AMPK activation, and mtROS production could predict

the various metabolic states in the cancer cells. All these highlight the critical role of redox homeostasis in the maintenance of a hybrid metabolic state of cancer cells (20).

To understand the unusual redox phenotype, Ren *et al.*, investigated the heterogeneity in cancer cell metabolism upon GPx2 knockdown in mice tumors by single-cell RNA-sequencing. While the majority of cancer cell clusters (6/7) exhibited elevated glycolytic gene signature, only cluster 5 showed elevation of both glycolytic and OXPHOS gene expression, the characteristic of hybrid metabolic state (*Figure 1*) suggesting that, cluster 5 which has a hybrid metabolic status may survive in both aerobic and hypoxic conditions. The hybrid metabolic state in cluster 5 was further confirmed by co-immunostaining of tumor sections with GLUT1 and phospho-AMPK (4). Though this type of subpopulation might be the attractive therapeutic target, it is not clear about the prevalence of this metabolic hybrid phenotype that contributes to the total cell population. Since the metabolic readout of the hybrid population is masked in the bulk phenotype of the cells, it is crucial to establish the proportion and functional metabolic phenotype of hybrids. Moreover, co-immunostaining of metabolic hybrid subtypes (GLUT1+ pAMPK+) in primary and metastatic tissues with hypoxia and ROS markers, would help establish a quantitative link between ROS levels, HIF-1 α , and hybrid state.

One major caveat of the study is reliance on transcriptional metabolic signatures to identify the metabolic phenotypes in single-cell sequencing data, and the lack of functional validation. To date, there are no established markers to isolate and characterize these metabolic hybrids for their functional validation *ex vivo*. Sorting of these subpopulations would not only help validate metabolic hybrids but also identify novel therapeutic targets boosting the research in the field.

Another major limitation of the study is the characterization of metabolic hybrids in the metastatic niches. The pathway analysis of metabolic hybrids in cluster 5 shows enrichment of EMT genes suggestive of metastasis (4). Although the link between metabolic hybrids and metastasis is correlated based on associative data and previous mathematical modeling-based studies, in-depth analysis using scRNA-seq would have been insightful. It is unclear if the metabolic hybrids are enriched in the metastatic lesions compared to the primary tumor and if there is a loss of metabolic hybrids in GPx2 knockdown tumors upon treatment with N-acetylcysteine or HIF-1 α inhibitors.

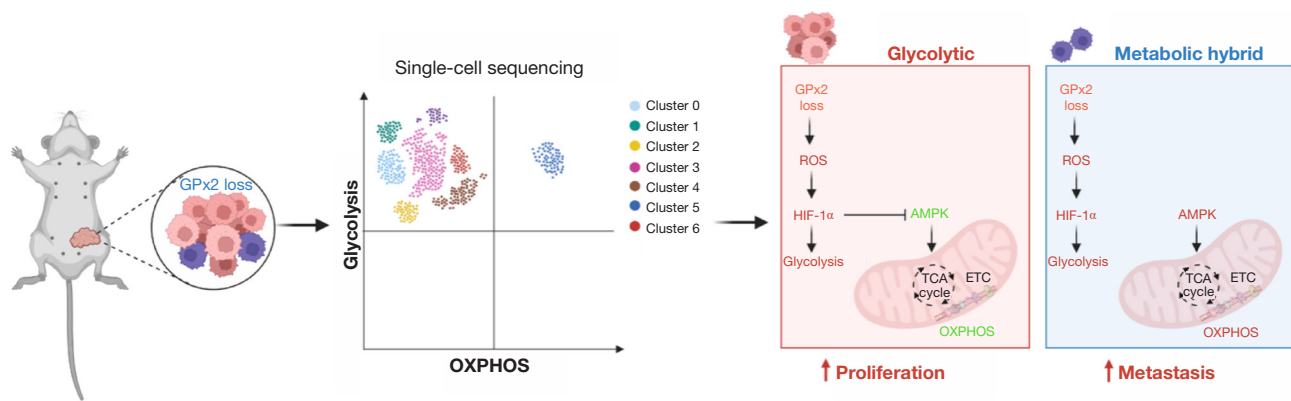


Figure 1 Illustration of GPx2-regulated hybrid metabolic phenotype using hypothetical data developed from the observations of Ren *et al.* (4). Implantation of GPx2 knockdown PyMT1 breast cancer cells in mice leads to metabolic heterogeneity in cancer cells. Single-cell sequencing profiles separates major glycolytic phenotypes and a minor hybrid metabolic phenotype. GPx2 knockdown cells with higher glycolysis and reduced OXPHOS (glycolytic) contribute to active proliferation. However, a small population of hybrid metabolic cells with elevated both glycolysis and OXPHOS is more relevant in metastatic progression. The upregulated genes/pathways are depicted in red while downregulated are shown in green. The figure was created by BioRender.com. GPx2, glutathione peroxidase 2; ROS, reactive oxygen species; TCA, tricarboxylic acid; ETC, electron transport chain; OXPHOS, oxidative phosphorylation.

FAO is another critical node intricately associated with the HIF-1 α :AMPK:ROS circuit in the hybrid metabolic state (20). Apart from glucose oxidation (GO), FAO generates the common intermediary metabolite, acetyl CoA that feeds the TCA cycle to drive OXPHOS. Interestingly, both FAO and GO metabolic and gene signatures were enriched in the hybrid metabolic state from invasive breast cancer samples. In this model, hybrid metabolic cells were predicted to thrive in moderate ROS to support the high energy demand in the cells. Further, dual inhibition of glycolysis and FAO targeting metabolic hybrids inhibited the growth of metastatic breast cancer cells (20). Moreover, inhibition of FAO by etomoxir drastically reduced the invasiveness, and metastatic potential of triple negative breast cancer in mice (14). Since GPx2 knockdown increased the ROS in metastatic breast cancer cells, it is intriguing to understand how FAO might play a role in supporting the hybrid metabolic state and reaffirming the role of ROS in metabolic plasticity.

Overall, this is an important study that highlights the tumor-promoting function of ROS upon GPx2 depletion in breast tumor proliferation, and metastasis associated with the emergence of a metabolically hybrid tumor state. However, the exact relation between high ROS levels and metabolic hybrid phenotype is not clear at this stage. Importantly, the study raises a very crucial question in the field if the chemotherapy-induced high levels of ROS

shape the pro- or anti-tumor function and if the metabolic hybrid state plays any role in this? Future studies directed towards the isolation and functional characterization of these metabolically hybrid cell clusters would help to decipher their significance in adaptation to various tumor microenvironments.

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to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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