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Disrupting the 'Warburg effect' re-routes cancer cells to OXPHOS offering a vulnerability point *via* 'ferroptosis'-induced cell death

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ABSTRACT

The evolution of life from extreme hypoxic environments to an oxygen-rich atmosphere has progressively selected for successful metabolic, enzymatic and bioenergetic networks through which a myriad of organisms survive the most extreme environmental conditions. From the two lethal environments anoxia/high O_2, cells have developed survival strategies through expression of the transcriptional factors ATF4, HIF1 and NRF2. Cancer cells largely exploit these factors to thrive and resist therapies.

In this review, we report and discuss the potential therapeutic benefit of disrupting the major Myc/Hypoxia-induced metabolic pathway, also known as fermentative glycolysis or "Warburg effect", in aggressive cancer cell lines. With three examples of genetic disruption of this pathway: glucose-6-phosphate isomerase (GPI), lactate dehydrogenases (LDHA and B) and lactic acid transporters (MCT1, MCT4), we illuminate how cancer cells exploit metabolic plasticity to survive the metabolic and energetic blockade or arrest their growth. In this context of NRF2 contribution to OXPHOS re-activation we will show and discuss how, by disruption of the cystine transporter xCT (SLC7A11), we can exploit the acute lethal phospholipid peroxidation pathway to induce cancer cell death by 'ferroptosis'.

1. Introduction

In the past decade, there has been a growing interest in cancer metabolism, particularly glucose and glutamine metabolism in cancer cells. These topics have now become an integral part of cancer biology in a similar realm to signal transduction and transcription. Contrasting to normal differentiated cells that derive their energy from respiration (OXPHOS), it is now well documented that most rapidly developing tumors depend primarily on fermentative glycolysis even when oxygen is plentiful, a phenomenon referred to as the "Warburg effect" (Hay, 2016; Kroemer and Pouyssegur, 2008; Vander Heiden et al., 2009; Warburg, 1956). This high glycolytic phenotype, also referred to as 'glycolytic addiction', results from the conjunction of uncontrolled growth signaling, deregulated c-Myc and hypoxia-induced Factor 1 (HIF-1) activity leading to induction of the glycolytic enzymes (Brahimi-Horn et al., 2011; Hsieh et al., 2015; Hubbi and Semenza, 2015) and inhibition of pyruvate oxidation in mitochondria (Chae et al., 2016; Kim et al., 2006). This accelerated aerobic glycolysis has been known to distinguish cancer cells from normal cells for many decades and this key feature has been exploited to detect and image tumors *in vivo*.

Despite the remarkable genetic and phenotypic tumor heterogeneity, a specific set of signaling pathways appear to support the

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altered metabolic processing of glucose and essential nutrients. Indeed, there is a dual set of universal mitogenic pathways: Ras-Raf-ERK and PI3K-AKT, activated by growth factors/hormone receptor tyrosine kinases and G protein-coupled receptors. ERKs and AKTs protein kinases synergize in controlling growth and metabolism through activation of the evolutionary conserved master protein kinase mTORC1 (Gonzalez and Hall, 2017; Laplante and Sabatini, 2012). In cancer, oncogenes and tumor suppressors constitutively activate these mitogenic pathways through Myc and HIF1 to promote metabolism and biomass accumulation and allow cancer cells to cope with multiple nutritional and oxidative stresses through increased expression of ATF4 (Broer and Broer, 2017; Wortel et al., 2017) and NRF2 (Taguchi and Yamamoto, 2017).

The rather common 'glycolytic' cancer phenotype has prompted many studies to investigate whether specific inhibition of glycolysis in tumors may have clinical benefit. Initial studies have exploited inhibition of glycolysis, with 2-deoxy-glucose (2-DG), a competitive inhibitor of glucose transport, and end product inhibitor of hexokinases and glucose-6-phosphate isomerase (Hay, 2016; Pouyssegur et al., 1980a; Pusapati et al., 2016) or with the alkylating metabolic inhibitor 3-bromopyruvic acid (Birsoy et al., 2013; Pedersen, 2007). High toxicity of these inhibitors has greatly limited their use in the clinic as they both inhibit the dual metabolic flux of glycolysis and OXPHOS. Other investigators have instead explored inhibition, gene silencing or disruption of specific downstream steps of glycolysis, namely lactate dehydrogenases A and B (Boudreau et al., 2016; Brand et al., 2016; Fantin et al., 2006; Le et al., 2010) or the ultimate step of exporting lactic acid *via* the H^+/lactate$^-$ symporters (MCT1 and MCT4) (Doherty et al., 2014; Granja et al., 2015; Le Floch et al., 2011; Marchiq et al., 2015).

Here we discuss the benefits and limitations of genetically disrupting the 'Warburg effect' at three levels of the glycolytic pathway: i) an upstream block at the level of the glucose-6-phosphate isomerase (GPI), ii) a downstream block at the level of lactate dehydrogenases (LDH, isoforms A and B) and iii) preventing lactic acid export via H^+/lactate$^-$ transporters (MCT1 and 4). Using these three examples of glycolysis genetic disruption studied in our lab, we discuss the responses of different cancer cell lines in terms of metabolic re-wiring, growth arrest and tumor escape and compare it with the broader literature. Finally, considering cancer metabolic re-wiring towards OXPHOS and subsequent increased oxidative stress, we discuss 'ferroptosis', a rather novel cell death mechanism as a possible anticancer strategy through inhibition of the cystine transporter xCT (SLC7A11), a guardian of the antioxidant defense pathway (Conrad et al., 2016; Dixon et al., 2012).

2. Disruption of glucose 6-phosphate isomerase (GPI) in cancer

GPI (D-glucose-6-phosphate aldose-ketose-isomerase; EC 5.3.1.9) is a ubiquitously expressed housekeeping cytosolic enzyme that catalyzes the reversible inter-conversion between glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) in glycolytic and gluconeogenic pathways (Fig. 1A). Like most glycolytic genes, its expression is directly induced by c-Myc (Kim et al., 2004) and HIF-1 (Funasaka et al., 2005) and as such plays a key role in many cancers (Pusapati et al., 2016).

More than 30 years ago, a *GPI*-defective mutant was isolated from Chinese hamster fibroblasts using [^{3}H]2-DG radiation suicide as a selection method (Pouyssegur et al., 1980a). As a consequence of the glycolysis disruption, these cells became exclusively dependent on respiration for energy production and growth. Despite glycolysis disruption, these cells retained the *in vitro* transformed phenotype of the parental line - low serum dependence and loss of anchorage dependence for growth, suggesting that (i) glycolysis is not categorically required for growth and (ii) aerobic glycolysis associated with malignant transformation is dispensable for the expression of the *in vitro* transformed phenotype. Furthermore, *GPI* mutation did not prevent initiation of DNA synthesis or tumor-forming capability even if these tumors developed more slowly, which might be explained by their higher sensitivity to hypoxia (Pouyssegur et al., 1980a, 1980b).

In our lab, we achieved complete genetic ablation of *GPI* expression using the CRISPR/Cas9 technique in two aggressive cancer cell lines, human colon adenocarcinoma (LS174T) and mouse melanoma (B16-F10) (de Padua et al., 2017). We showed that both *GPI*-KO cell lines had no measurable GPI enzymatic activity, no detectable secretion of lactic acid and display only a 2-fold reduced growth rate in normoxia (de Padua et al., 2017). These findings were in agreement with our early studies (Pouyssegur et al., 1980b) but diverged drastically from pharmacological inhibition of the glycolytic pathway suggesting that most of the drugs reported so far had off-target effects. In order to compensate for 'Warburg effect' disruption, *GPI*-KO cells re-directed their bioenergetic needs towards the Pentose Phosphate Pathway (PPP) linked to OXPHOS as seen by the increased respiratory capacity (de Padua et al., 2017). The vulnerability of this metabolic rewiring is an increased reliance on oxygen. Indeed *GPI*-KO cells, although remaining fully viable, cannot grow in hypoxia (1% O_2) and become extremely sensitive to inhibitors of respiratory chain complexes, such as phenformin and oligomycin (de Padua et al., 2017). These results are in line with the findings reported for several pancreatic cancer cell lines by Pusapati et al. (2016). Interruption of the glycolytic flow by *GPI* ablation leads to accumulation of its substrate, intracellular G6P, which in turn was proposed to elicit a short-term inhibition of hexokinase and a long-term inhibition of glucose transport (Pouyssegur et al., 1980a; Ullrey et al., 1982). Indeed, we found decreased GLUT1 expression in both *GPI*-KO cell lines, as well as induction of Thioredoxin Interacting Protein (TXNIP), which is known to impose a strong negative regulation on glucose uptake (Stoltzman et al., 2011; Wu et al., 2013).

Finally, we showed that *GPI*-KO and subsequent suppression of the 'Warburg effect' in two aggressive cancer cell lines only slowed down, but did not prevent *in vivo* tumor growth, in line with the early and more recent findings (Pouyssegur et al., 1980a; Pusapati et al., 2016). Particularly striking is the example of LS174T, a highly glycolytic cancer cell line, that under normal conditions almost does not respire on glucose, and is capable of strong re-activation of OXPHOS when challenged by *GPI* ablation. Therefore, it is clear that pharmacological inhibition of glycolysis and tumor growth reported previously with 2-DG or 3-Bromopyruvate was mainly effective because of their multiple targets affecting both glycolysis and OXPHOS. Consequently, as shown with inducible shRNAs against *GPI* (Pusapati et al., 2016) tumor growth was significantly reduced only in combination with mTORC1 or OXPHOS inhibition. After this example of *GPI* genetic disruption, an early step of the glycolytic pathway, we explore in the next two sections whether metabolic re-routing and cancer growth can be generalized to downstream ablation of the glycolytic flux.