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Defining the Molecular Basis of Tumor Metabolism: a Continuing Challenge Since Warburg's Discovery

Ana Carolina Santos de Souza[1], Giselle Zenker Justo[2], Daniele Ribeiro de Araújo[1] and Alexandre D. Martins Cavagis[3]

[1]Centro de Ciências Naturais e Humanas (CCNH), Universidade Federal do ABC (UFABC), Santo André, [2]Departamento de Ciências Biológicas (*Campus* Diadema) and Departamento de Bioquímica (*Campus* São Paulo), Universidade Federal de São Paulo (UNIFESP), São Paulo, [3]Departamento de Física, Química e Matemática (DFQM), Universidade Federal de São Carlos (UFSCar), *Campus* Sorocaba, Sorocaba

Key Words

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Abstract

Cancer cells are the product of genetic disorders that alter crucial intracellular signaling pathways associated with the regulation of cell survival, proliferation, differentiation and death mechanisms. The role of oncogene activation and tumor suppressor inhibition in the onset of cancer is well established. Traditional antitumor therapies target specific molecules, the action/expression of which is altered in cancer cells. However, since the physiology of normal cells involves the same signaling pathways that are disturbed in cancer cells, targeted therapies have to deal with side effects and multidrug resistance, the main causes of therapy failure. Since the pioneering work of Otto Warburg, over 80 years ago, the subversion of normal metabolism displayed by cancer cells has been highlighted by many studies. Recently, the study of tumor metabolism has received much attention because metabolic transformation is a crucial cancer hallmark and a direct consequence of disturbances in the activities of oncogenes and tumor suppressors. In this review we discuss tumor metabolism from the molecular perspective of oncogenes, tumor suppressors and protein signaling pathways relevant to metabolic transformation and tumorigenesis. We also identify the principal unanswered questions surrounding this issue and the attempts to relate these to their potential for future cancer treatment. As will be made clear, tumor metabolism is still only partly understood and the metabolic aspects of transformation constitute a major challenge for science. Nevertheless, cancer metabolism can be exploited to devise novel avenues for the rational treatment of this disease.

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Introduction

Cancer arises when cells undergo uncontrolled proliferation through enhanced activity of oncogenes and reduced activity of tumor suppressors. Normal cells undergo six major alterations to become tumor cells,

Ana Carolina Santos de Souza
Centro de Ciências Naturais e Humanas (CCNH)
Universidade Federal do ABC (UFABC) Santo André, SP (Brasil)
Tel. +55-11- 4996 7960, Fax +55-11-4996 0090
E-Mail ana.galvao@ufabc.edu.br

namely, "self-sufficiency" in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [1]. An additional alteration, the subversion of normal cell metabolism, has recently been identified as another important hallmark of cancer cells [2].

Although the unique characteristics of tumor cell metabolism have been studied since the pioneering work of Nobel Prize winner Otto Warburg over 80 years ago [3], the relative importance of this metabolism for tumor onset and growth is only now gaining attention. Recent studies have demonstrated the essential role of glucose and glutamine in maintaining the appropriate function of key metabolic pathways and their contribution to metabolic transformation in cancer cells. Considering the relative inefficiency of antitumor therapies in targeting the six canonical hallmarks of cancer mentioned above, a better knowledge of cancer cell metabolism would certainly provide new possibilities for cancer treatment and an important improvement in traditional therapeutic strategies.

In this review, we summarize the current scenario of this new field of cancer biology, generally referred to as the Warburg effect. In particular, we discuss the relative contribution of glycolysis and oxidative phosphorylation in supplying the energy requirements of normal proliferating cells and tumor cells and the new findings about the central role of the tricarboxylic acid (TCA) cycle, pentose phosphate pathway and glutaminolysis in cancer cell growth. In addition, recent discoveries on the effects of oncogenes and tumor suppressors in controlling specific metabolic pathways and the overall metabolic control associated with growth signaling pathways are reviewed.

The Warburg effect

In the 1920s, Otto Warburg published the seminal observation that rapidly proliferating ascites tumor cells consume glucose at a surprisingly high rate compared to normal cells. Additionally, Warburg found that even at normal O_2 tension these cells fermented glucose into lactate rather than oxidizing it completely, a phenomenon known as the Warburg effect [3-5]. Since this remarkable discovery, many reports have documented the Warburg effect in a variety of tumors, reinforcing Warburg´s observation that cancer cells use mainly glycolysis for generate energy [6, 7]. The functional rate of this "aerobic glycolysis" and lactate production correlates with

the degree of tumor malignancy, i.e., aerobic glycolysis is faster in highly de-differentiated and fast growing tumors than in slow-growing tumors or normal cells. In addition, a high glycolytic rate in tumor cells has been related to resistance to chemo- and radiotherapy [8, 9].

One of the possible explanations for the increase in tumor cell glycolytic rate is the overexpression of glucose transporters (GLUT) and of virtually all enzymes of the glycolytic pathway as a consequence of oncogene activation [10-12]. Accordingly, the high levels of glucose uptake in malignant cells have been associated with increased expression of glucose transporter proteins, such as GLUT1, GLUT3 and/or GLUT12 [11]. Furthermore, the overexpression and/or overactivation of hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK), the main enzymes controlling the glycolytic pathway, has been described for a number of tumors [10, 12, 13]. Interestingly, tumor cells have been suggested to use isoforms of glycolytic enzymes that differ from those used to drive glycolysis in normal cells, although this issue remains little studied and is controversial [14-20].

The distinctively higher levels of glucose uptake displayed by tumor cells compared to other tissues have been exploited clinically and are used to diagnose, monitor and treat cancer. For example, with positron emission tomography (PET), which uses ^{18}F-deoxyglucose as a glucose analogue and tumor marker, it is possible to detect and gauge the size of a tumor before and after anticancer therapy. Additionally, PET allows the tracking of metastasis with an accuracy >90%. These findings indicate the close relationship between the Warburg effect and the invasiveness and metastatic potential of cancer cells [21].

In addition to their direct clinical applications and contribution to the study of cancer biology, Warburg's observations also raised some disturbing questions. For example, considering that the metabolism of glucose to lactate generates only two ATPs per molecule of glucose whereas oxidative phosphorylation generates up to 36 ATPs through complete oxidation of one glucose molecule, why do cancer cells, which have a high rate of proliferation and, consequently, consume a large amount of ATP, "choose" to oxidize glucose partially through aerobic glycolysis rather than completely oxidizing this compound through mitochondrial metabolism? And why do cancer cells, even in the presence of sufficient oxygen, "prefer" to obtain the ATP necessary for growth and proliferation through a less efficient form of metabolism (in terms of the number of ATP molecules produced)?

One explanation for the apparent preference of cancer cells for the glycolytic pathway was provided by Warburg himself. His original hypothesis proposed that cancer originated from irreversible damage to mitochondrial respiration followed by an increase in glycolysis to replace the ATP lost from defective oxidative phosphorylation. This shift from oxidative phosphorylation to glycolysis turns highly differentiated cells into undifferentiated cells that proliferate as cancer cells [6, 22]. However, since Warburg´s original discovery, many studies have demonstrated that in most tumors mitochondria are not dysfunctional and that oxygen consumption by these cells is not reduced when compared to their non-tumor counterparts [13, 23, 24]. Additionally, normal proliferating cells also reprogram their metabolism to fuel the simultaneous need for growth and proliferation through increased rates of glycolysis, even under normoxic conditions and in the presence of functional mitochondria (see reference 22 for an excellent review of metabolism in normal proliferating cells). Together, these findings demonstrate that the Warburg effect frequently develops independently of the state of mitochondrial function and that metabolic reprogramming is not limited to tumor cells but, rather, is a common metabolic switch occurring in all proliferating cells, with tumor cells having a higher level of glycolysis compared to their normal proliferating counterparts.

The glycolytic pathway: fuelling cellular growth and proliferation with ATP and macromolecular precursors

As indicated above, it is strange that the glycolytic pathway, rather than mitochondrial metabolism, should predominate in normal highly proliferating cells and cancer cells, particularly in view of the relative inefficiency of glycolysis in completely oxidizing glucose and the large amount of energy required to drive anabolic processes during cell growth and proliferation. However, inefficient ATP production by glycolysis is apparently a problem only when nutritional resources are scarce. Recent studies of metabolic pathways and their regulation in proliferating cells have shown that, in the presence of abundant nutrients, anaerobic glycolysis provides cells with high ratios of ATP/ADP and NADH/NAD$^+$, regardless of how much these cells are stimulated to divide [15, 22, 25]. In addition to a large amount of ATP, growing and proliferating cells also require a means of rapidly producing this energy. In this regard, the high rates of glycolysis observed in normal proliferating cells and cancer cells provide an appropriate means of producing ATP to meet the anabolic and bioenergetic requirements. Indeed, when glucose is in excess, glycolysis can potentially produce ATP in greater amounts and faster than mitochondrial oxidative phosphorylation [22, 26, 27]. A parallel situation is observed in fermenting yeasts which grow at higher rates than those that use oxidation-based metabolic processes [28], indicating that high glycolytic rates can boost cell growth and proliferation.

A second advantage of the high glycolytic rate in normal proliferating cells and cancer cells is related to the high demand for NADPH and molecular intermediates to sustain the continuous synthesis of macromolecular building blocks needed to drive the increase in cellular biomass and duplication of genetic material. Cell growth requires more equivalents of carbon and NADPH than ATP to sustain lipid, amino acid and nucleotide biosynthesis. Consequently, proliferation and growth can be sustained by processes that not only produce ATP but also generate a large amount of reducing power and metabolic intermediates required by anabolic pathways. In this regard, aerobic glycolysis represents an adequate metabolic pathway for growing and proliferating cells since, at high rates, it provides cells with ATP and glycolytic intermediates that are an important source of precursors for the synthesis of non-essential amino acids, lipids and nucleic acids. In addition, the accumulation of glycolytic intermediates can stimulate the oxidative and non-oxidative arms of the pentose phosphate pathway to generate, respectively, NADPH and ribose-5-phosphate for nucleic acid biosynthesis [6]. Importantly, the increased rate of NADPH production also provides a reducing environment for the anabolic synthesis of biomolecules such as fatty acids and cholesterol (Fig. 1).

The high rate of glycolysis in proliferating cells can also help to protect against cellular oxidative damage. The increased levels of intracellular NADPH generated through stimulation of the pentose phosphate pathway by glycolytic intermediates lead to an increase in the reduced form of glutathione (GSH), a major non-enzymatic antioxidant. Glycolysis may therefore have an important role in maintaining the integrity and functionality of biomolecules during the enhanced biosynthesis of macromolecules and genetic material in proliferating cells. The increased levels of reduced GSH may also help to detoxify antineoplastic drugs or antagonize their effects. Indeed, higher glycolytic rates are associated with more aggressive and resistant tumors [21, 29].
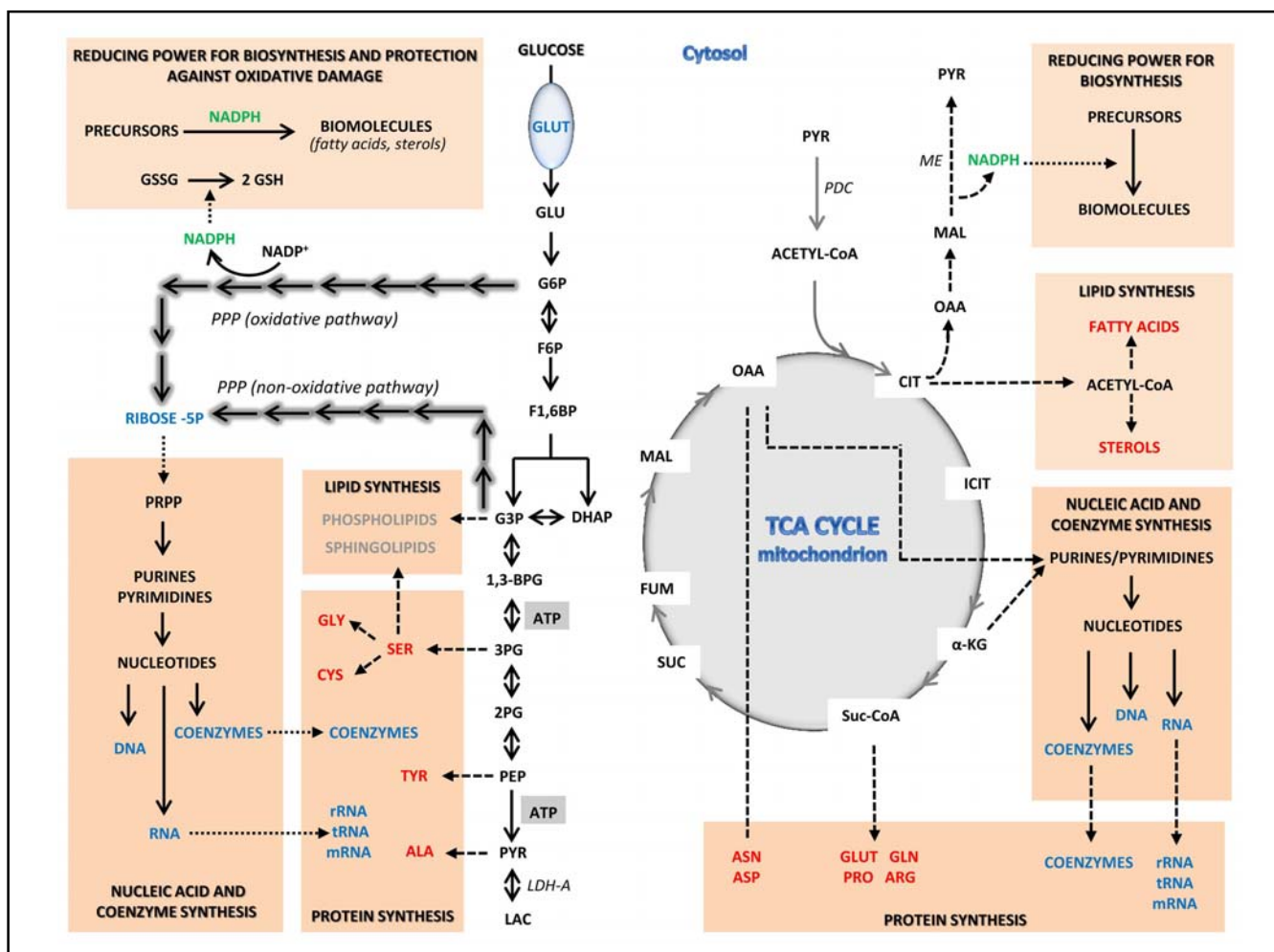
Fig. 1. The importance of glycolysis and the TCA cycle for cancer cells. A high glycolytic rate supports tumor growth and proliferation. In the presence of abundant glucose, the glycolytic pathway provides tumor cells with the ATP and macromolecular precursors necessary to supply their bioenergetic and anabolic requirements. Furthermore, the high use of glucose by the glycolytic pathway stimulates the pentose phosphate pathway, leading to an increase in ribose 5-phosphate levels and NADPH production. Cancer cells can use ribose 5-phosphate to synthesize nucleotides required for the duplication of genetic material, RNA synthesis and protein translation. The enhanced production of NADPH is a crucial event since this compound provides the reducing power for macromolecule biosynthesis while at the same time protecting these macromolecules against oxidative stress. Although the role played by the TCA cycle in energy production by cancer cells is uncertain, this cycle has an important function as a source of molecular precursors for the synthesis of biomolecules. 1,3-BPG: 1,3-bisphosphoglycerate; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; ALA: alanine; ARG: arginine; ASN: asparagine; ASP: aspartate; CIT: citrate; CYS: cysteine; DHAP: dihydroxyacetone phosphate; F1,6BP: fructose 1,6-bisphosphate; F6P: fructose-6-phosphate; FUM: fumarate; G3P: glyceraldehyde 3-phosphate; G6P: glucose-6-phosphate; GLN: glutamine; GLU: glutamate; GLU: glucose; GLUT: glucose transporter; GLY: glycine; GSH: reduced form of glutathione; GSSG: oxidized form of glutathione; ICIT: isocitrate; LDH-A: lactate dehydrogenase A; MAL: malate; ME: malic enzyme; mRNA: messenger RNA; OAA: oxaloacetate; PDC: pyruvate dehydrogenase complex; PEP: phosphoenolpyruvate; PPP: pentose phosphate pathway; PRO: proline; PRPP: phosphoribosylpyrophosphate; PYR: pyruvate; rRNA: ribosomal RNA; SER: serine; SUC: succinate; Suc-CoA: succinyl-CoA; tRNA: transfer RNA; TYR: tyrosine; α-KG: α-ketoglutarate.

The functions of glycolytic enzymes are not restricted to the glycolytic pathway since these enzymes are also involved in non-glycolytic functions that contribute to tumor development, survival and, importantly, resistance to cell death. Thus, for example, hexokinase II (HK2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

and lactate dehydrogenase (LDH) have non-glycolytic functions that confer relative advantages to cancer cells. GAPDH and LDH are incorporated into the transcriptional factor complex OCA-S, which increases histone transcription (H2B gene) and favors tumor growth. GAPDH also interacts with nucleic acids and participates in transcriptional regulation (as a nuclear tRNA export protein and regulator of mRNA stability) and DNA replication and repair [30-33]. The embryonic isoform of PK, known as pyruvate kinase M2 (PK-M2), can translocate to the nucleus where it participates in the phosphorylation of histone 1. PK-M2 may also modulate transcription factors, such as Oct4, that play an important role in preventing the expression of genes associated with differentiation [34]. The glycolytic enzyme phosphoglycerate kinase (PGK) is secreted by tumor cells and acts as a disulfide reductase that facilitates the cleavage of disulfide bonds in plasmin, thereby triggering proteolytic release of the angiogenesis inhibitor, angiostatin [35].

Wartenberg et al. [36] recently described an association between high glycolytic metabolism and increased expression of P-glycoprotein (P-gp). P-gp is a member of the ABC (ATP binding cassette) transporters, a family of transmembrane proteins that act as efflux pumps and efficiently remove structurally unrelated chemotherapeutic drugs from tumor cells, thereby lowering the intracellular drug concentration below the effective dose, in a phenomenon known as multidrug resistance (MDR). MDR is currently considered the main obstacle in effective cancer therapy since acquisition of the MDR phenotype by cancer cells prior to or during therapy is responsible for the failure of most antineoplastic therapies in eradicating the disease. These findings suggest that the inhibition of glycolysis could be used to reverse the MDR phenotype and improve traditional antineoplastic therapies.

High glycolytic rates confer resistance to death

As stated above, high levels of glycolysis are associated with increased resistance of cancer cells to cell death, including that induced by therapeutic agents. Hence, understanding the molecular basis of this relationship is fundamental for improving current antitumor therapies and for developing more effective death-inducing drugs. Recent studies have contributed to this goal by demonstrating that the non-enzymatic

functions of glycolytic enzymes and/or the accumulation of key glycolytic intermediates can affect the normal mitochondrial physiology, inducing a phenotype of increased resistance to cell death [37-39].

One of the glycolytic enzymes whose functions extend beyond glycolysis is HK2. This enzyme catalyzes the first step of glycolysis and is highly expressed in transformed cells. Curiously, in cancer cells, over 70% of HK2 is bound to mitochondria, indicating the existence of an alternative role for this enzyme besides its classic function in the glycolytic pathway. In agreement with this conclusion, Pastorino et al. [40] demonstrated that HK2 competes with Bcl2 family proteins for binding to the voltage-dependent anion channel (VDAC) to influence the balance of pro- and anti-apoptotic proteins that control permeabilization of the outer mitochondrial membrane. During apoptosis, pro-apoptotic proteins of the Bcl2 family, such as Bax and Bak, oligomerize in the outer mitochondrial membrane to form a channel through which cytochrome c is released. Once in the cytosol, cytochrome c associates with other proteins to form the apoptosome, which is responsible for the execution phase of apoptosis by stimulating caspase activation. The binding of HK2 to the VDAC displaces the anti-apoptotic protein Bcl-XL and makes it available for interaction with Bax and Bak, thereby inhibiting their pro-apoptotic actions that can contribute to outer membrane permeabilization. The binding of HK2 to VDAC also affects channel permeability, leading to a closed state that inhibits cytochrome c release. In addition, HK2 antagonizes the pro-apoptotic effects of the protein Bid, which is responsible for the activation of Bax and Bak [38].

The involvement of high glycolytic rates in the resistance of cancer cells to apoptosis is also demonstrated by the effect of glycolytic intermediates on mitochondrial structure. Accelerated glucose metabolism leads to a predominance of reduced cytochrome c over its oxidized form, and reduced cytochrome c is unable to trigger cell death despite being released from the mitochondrial intermembrane space. This response is strongly related to the repression of oxidative metabolism in cancer cells exposed to high glucose concentrations, a phenomenon known as the Crabtree effect [41]. The precise mechanism by which the Crabtree effect is triggered is unknown, although several mechanisms have been proposed to explain its function. Diaz-Ruiz et al. [37] demonstrated that the glycolytic intermediate fructose 1,6-biphosphate inhibits the activity of cytochrome c oxidase, leading to inhibition of the mitochondrial respiratory chain and a decrease in

respiration; this action favors the maintenance of cytochrome c in its reduced state in which is unable to trigger programmed cell death. This observation provides a rational explanation for the association between glycolysis, the Crabtree effect and apoptosis repression, namely, that the inhibitory action of the high glycolytic rate of tumor cells on mitochondrial respiration may serve to protect against cell death by reducing the production of reactive oxygen species (ROS) and inhibiting cytochrome c-induced cell death [37].

The various aspects discussed above clearly indicate that the resistance of cancer cells to death is strongly related to mitochondrial structure and function. Increased glycolytic activity and the overexpression of glycolytic enzymes can help cancer cells avoid death induced by anticancer drugs, either by altering the mitochondrial structures involved in the release of cytochrome c and apoptotic factors into the cytosol where they subsequently trigger apoptosis, or by inhibiting ROS formation through interference with the electron transport chain. These mechanisms could provide a basis for the inclusion of glycolysis-inhibiting drugs in the current arsenal of antitumor drugs.

The advantage of lactate secretion for cancer cells

The apparently inefficient ATP-producing glycolytic pathway benefits highly proliferating cells by increasing their rate of biomass formation. However, although the glycolytic pathway does offer advantages to proliferating cells, three carbon atoms are still lost in the form of lactate secreted by the cells. How can this loss of oxidizable carbons favor cancer cell growth and proliferation? An important consideration here involves the metabolic pathways active in specialized non-proliferating tissues that recycle the excess lactate and alanine released by rapidly proliferating cells [25]. Tumor cells can take advantage of the lactate secreted by other cells in specific situations since cellular metabolism within a tumor is usually heterogeneous, especially in growing tumor masses where the oxygen and nutrient supply by the blood decreases in central regions as tumor volume increases. An interesting "metabolic symbiosis" has been proposed between well-oxygenated (aerobic) and poorly oxygenated (hypoxic) cancer cells within the tumor mass. Hypoxic cells are characterized by a large demand for glucose uptake and a high lactate release via the monocarboxylate transporter 1 (MCT1). Normoxic cells can, in turn, take up the secreted lactate through other MCTs, such as MCT4, and convert it to pyruvate, which fuels mitochondrial metabolism. Through this mechanism, peripheral cancer cells, which have access to major nutrients and oxygen from blood, can meet their energy requirements by oxidizing pyruvate derived from the lactate secreted by hypoxic cells. This use of pyruvate reduces the uptake of blood glucose by normoxic cells, leading to a higher glucose concentration in the blood reaching the hypoxic central regions of the tumor. This greater glucose availability in turn enhances the survival of hypoxic tumor cells, which obtain their energy requirements for growth and survival solely through anaerobic glycolysis [42].

The secretion of lactate by plasma membrane MCTs (which co-transport H^+ with lactate) has been related to the maintenance of an acidic microenvironment that favors tissue invasion and metastasis. Cancer cells can also affect the extracellular pH by modulating the activity of the Na^+-H^+ exchanger, surface V-type H^+-ATPase and/or surface F_1F_0 ATPase and carbonic anhydrase isoforms 9 and 12 (CA9 and CA12). The increase in extracellular acidity may activate cathepsins and metalloproteinases, leading to the degradation of extracellular matrix and an increase in the susceptibility of the endothelial basal membrane to proteolytic attack [7, 21, 43]. The ability of cancer cells to increase the extracellular H^+ concentration in a variety of ways attests to the importance of this phenomenon in cancer invasiveness and metastasis.

The high glycolytic pathway activity responsible for the increased glucose consumption in cancer cells provides a fast way of meeting at least three requirements for rapid growth in proliferating cells, namely, an abundant energy supply, the availability of reducing power for the synthesis of biomolecules, and the formation of ribose-5-phosphate for nucleotide and nucleic acid biosynthesis. The rapid growth that accompanies the increased glucose uptake characteristic of the Warburg effect is directly associated not only with the high rate of glycolysis but also with stimulation of the pentose phosphate pathway. In agreement with this observation, certain tumor cells may grow in culture in the absence of glucose, as long as there are substrates to feed the pentose phosphate pathway. In addition, the metabolic adaptations of tumor metabolism include enhancement of the pentose phosphate pathway and a specific balance between the oxidative and non-oxidative branches to maintain the high proliferative rates [44-46].

Whereas the importance of a high rate of glycolysis in proliferating cells to meet the demands for biomass

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production and to protect newly synthesized molecules against oxidative damage through NADPH is clear, the real contribution of this metabolic pathway in supplying all of the energy requirements in cancer cells is still a matter of debate. This question is closely related to the current discussion about the functional state of the TCA cycle and mitochondrial oxidative phosphorylation in these cells. Indeed, the functional roles of the TCA cycle in these cells have been extensively debated, indicating the need for more studies in this area.

The anabolic and catabolic functions of the TCA cycle in cancer cells

Pyruvate: a scarce source of carbon atoms for the TCA cycle?

Although some tumors have defective mitochondria (as initially hypothesized by Warburg), in most tumor cells mitochondrial function remains normal [13, 47, 48]. An intriguing question is therefore why only about 10% of the pyruvate generated by the glycolytic pathway actually feeds into the TCA cycle and mitochondrial metabolism. What are the molecular mechanisms responsible for this observation?

Evidence for the limited delivery of pyruvate to mitochondria comes from observations that tumor cells selectively express PK-M2. Unlike other PK isoforms, PK-M2 is negatively regulated by tyrosine-phosphorylated proteins downstream from a variety of growth factor signals. PK-M2 can exist in either dimeric or tetrameric forms, which allows the enzyme to oscillate from the high activity form (tetrameric) to the low activity form (dimeric). When in its dimeric (low activity) form, the enzyme acts as a metabolic regulator that drives the flow of carbon into anabolic pathways, thereby avoiding its conversion to lactate or complete catabolism in mitochondria to generate ATP [15, 21, 49]. In tumor cells, PK-M2 occurs predominantly as a dimer with low activity, suggesting that its activity may be a target of oncogenes, many of which are tyrosine kinases that regulate growth factor signaling pathways [49, 50].

It is tempting to speculate that the low activity of PK-M2 in cancer cells is the key adaptation of tumor metabolism that limits the amount of pyruvate available for mitochondrial metabolism and determines the metabolic differences between these cells and untransformed cells. However, this apparent obstacle to the delivery of large amounts of pyruvate to mitochondria could be counterbalanced by the high rates of glucose

uptake and increased glycolysis characteristic of cancer cells. Other mechanisms (metabolic adaptations) may also be involved in deviating pyruvate from mitochondria thus stimulating its conversion to lactate. Two such mechanisms include the partial block of pyruvate transport to mitochondria and the overexpression of lactate dehydrogenase A (LDH-A). Indeed, in some cancer cells, the transport of pyruvate to mitochondria is slower than in non-tumor cells [51-54]. However, the question of whether pyruvate transport is diminished in tumor cells and the physiological consequences of this remain unanswered. The purification and characterization of the mitochondrial carrier for pyruvate would greatly enhance our limited knowledge of pyruvate transport.

In addition to a low level of pyruvate transport, another explanation for the low rates of pyruvate oxidation in mitochondria is the increased expression of the glycolytic enzyme LDH-A induced by oncogenes. LDH-A converts pyruvate to lactate, with the concomitant oxidation of NADH to NAD^+. Since NAD^+ is essential for glycolysis, the overexpression of LDH-A in tumor cells allows NADH to be quickly oxidized to NAD^+ in the cytosol, thereby enhancing the glycolytic flux under aerobic conditions [6]. This overexpression of LDH-A could contribute to the rapid conversion of pyruvate into lactate and to the diversion of most of the pyruvate generated by PK-M2 away from mitochondria.

An alternative or additional explanation for the high rates of glycolysis and lactate release in cancer cells is that these events are consequences of a massive production of pyruvate and reflect the inability of mitochondrial pathways to oxidize pyruvate rapidly enough to deal with the large amounts of this compound generated by glycolysis. Curiously, the pyruvate dehydrogenase complex (PDC), a group of enzymes responsible for the conversion of pyruvate into acetyl-CoA, is phosphorylated and has diminished activity in tumor cells. The action of the PDC commits the pyruvate molecule to complete oxidation via the TCA cycle or, alternatively, directs its carbon atoms to be used in the *de novo* synthesis of macromolecules. In normal proliferating cells, the glycolytic flux may exceed the PDC activity by more than one order of magnitude and, in this case, the conversion of pyruvate to lactate could prevent the intracellular accumulation of pyruvate and, consequently, the triggering of death mechanisms [22]. Whether a similar mechanism occurs in tumor cells remains to be determined.

In summary, it seems likely that tumor cell mitochondria are exposed to diminished amounts of

pyruvate. Consequently, these cells meet their energy requirements primarily through the glycolytic pathway. Interestingly, although some tumor cells have defective mitochondrial metabolic pathways, most of them depend upon the delivery of pyruvate to mitochondria and on the proper functioning of mitochondrial respiration to survive. In agreement with this, recent work by Thangaraju et al. [55] demonstrated the vital role of pyruvate transport to mitochondria in preventing colon cancer cell death. Thus, in addition to the mechanism that diverts pyruvate from further metabolism in mitochondria and the existence of mitochondria-defective tumors, it seems that the amount of pyruvate that reaches the TCA cycle in cancer cells is crucial for meeting the energy and/or anabolic requirements of these cells. Indeed, pyruvate is the main source of carbon atoms that, once incorporated into the TCA cycle in the form of acetyl-CoA, drives the *de novo* synthesis of lipids and proteins [56].

The TCA cycle as a source of metabolic substrates for the synthesis of biomolecules and cellular organelles

In order to proliferate, cancer cells need a large amount of energy and biomolecules as building blocks. An adequate supply of energy and biomolecules allows the cells to maintain their physiological functions and furnishes the anabolic pathways with ATP and substrates necessary for proliferation. The TCA cycle is essential for driving these events. Although the contribution of this cycle to ATP production by supplying the electron transport chain with reduced coenzymes is not exactly known, its central role as a source of metabolic intermediates for anabolic pathways (lipid, protein and nucleic acid biosynthesis) in growing cells is well-established (Fig. 1). The importance of the TCA cycle in proliferating cells is further illustrated by the fact that this pathway displays enhanced activity in a variety of tumor cells [57].

The first step in the TCA cycle is the formation of citrate from the condensation of acetyl-CoA and oxaloacetate, a reaction catalyzed by the enzyme citrate synthase. The rate of the TCA cycle is strongly associated with the relative amounts of these compounds. As mentioned, glycolytic pyruvate is the main source of acetyl-CoA, which is produced through catalysis by the PDC. Since the TCA cycle activity is increased in cancer cells it is likely that the amount of glucose-derived pyruvate delivered to mitochondria is high enough to supply the cycle´s requirements for acetyl-CoA. The TCA cycle has an interesting means of obtaining the oxaloacetate required to react with acetyl-CoA. In normal cells, increased

concentrations of acetyl-CoA are counterbalanced by the action of pyruvate carboxylase, an enzyme whose activity is allosterically regulated by the levels of acetyl-CoA; high levels of this compound stimulate pyruvate carboxylase and the conversion of pyruvate into oxaloacetate, thereby diverting pyruvate from the PDC and increasing the production of citrate. However, pyruvate carboxylase is suppressed in some tumor cells [20, 58, 59], raising questions as to how cancer cells can obtain the oxaloacetate essential for enhanced TCA cycle activity. The answer appears to be related to the high demand for glutamine by cancer cells [60].

In addition to glucose, glutamine is an important substrate for tumor cell growth and proliferation [60, 61]. The catabolism of glutamine may continuously supply the TCA cycle with α-ketoglutarate, allowing the generation of oxaloacetate and other metabolic intermediates required for biosynthetic pathways. At the same time, supplying glutamine to the TCA cycle stimulates the activity of this pathway and enhances mitochondrial respiration in tumor cells, in a response dependent on increased NADH and $FADH_2$ production [62, 63]. Furthermore, glutamine-dependent transamination can provide nitrogen for the synthesis of non-essential amino acids, and glutaminolysis (the conversion of glutamine into lactate), like the metabolism of glucose via the pentose phosphate pathway, produces NADPH via malic enzyme, a $NADP^+$-specific malate dehydrogenase (Fig. 2). Recent studies suggest that the amount of NADPH supplied by malic enzyme for cellular metabolism is the same as that provided by the pentose phosphate pathway, further highlighting the importance of glutamine in generating the redox potential required for anabolic processes in cell growth [56, 64].

Finally, the diversion of glutamine-derived malate from pyruvate to oxaloacetate, concomitant with the generation of acetyl-CoA from pyruvate through the PDC, also contributes to citrate production in the first step of the TCA cycle. These reactions initiate the oxidation of glutamine and glucose carbons in order to generate ATP or, alternatively, can be the starting point for the synthesis of macromolecule precursors, such as those associated with lipid synthesis in the cytosol. During the synthesis of fatty acids, the carbons from glucose and glutamine are exported from the mitochondrial matrix to the cytosol in the form of citrate. In the cytosol, citrate is converted to acetyl-CoA and oxaloacetate by the action of ATP-citrate lyase (ACL) in a reaction that requires free energy from ATP hydrolysis. The acetyl-CoA generated is used to extend the fatty acid acyl chains
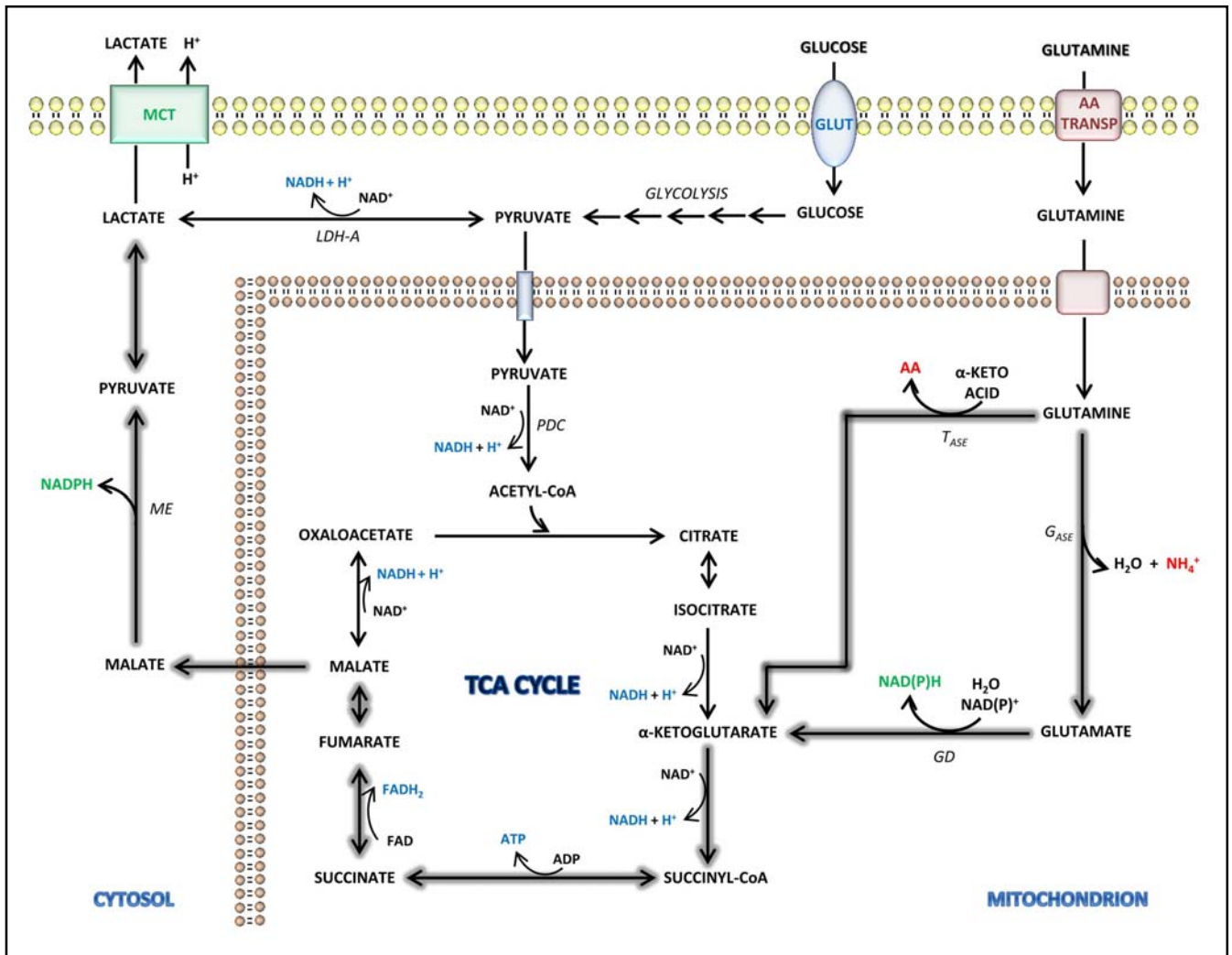
Fig. 2. Glutamine boosts the TCA cycle function in cancer cells. Glutamine, the most abundant amino acid in the blood, supplies the TCA cycle by generating α-ketoglutarate. The increased availability of this intermediate allows its conversion to malate, which can be transported to the cytosol where it is converted into pyruvate and then into lactate. During this process, known as glutaminolysis, the intermediates formed can be used to synthesize macromolecules necessary for tumor growth. In addition, the malate produced in response to glutamine can be diverted from lactate production in the cytosol to its oxidation in subsequent reactions of the TCA cycle, thereby contributing to the synthesis of ATP and reduced coenzymes, the latter being a stimulator of the electron transport chain. The term "truncated" TCA cycle reflects the fact that part of the citrate produced is diverted from the pathway to the synthesis of lipids. AA: amino acid; AA transp: amino acid transporter; Gase: glutaminase; GD: glutamate dehydrogenase; GLUT: glucose transporter; LDH-A: lactate dehydrogenase A; MCT: monocarboxylate transporter; ME: malic enzyme; PDC: pyruvate dehydrogenase complex; Tase: transaminase.

through a series of reactions that consume NADPH as a reducing source.

Another important issue related to the TCA cycle is the relative extent to which tumor cells use the carbon atoms derived from glucose and glutamine to drive the synthesis of biomolecules or energy (in the form of ATP). Some cancer cells show a considerable citrate efflux from mitochondria and appear to have a "truncated"

TCA cycle feeding the production of cholesterol, fatty acids and other products [56, 65, 66]. However, there is also evidence for an apparently normally functioning TCA cycle in a variety of other tumor cells that appear to depend on mitochondrial metabolism for their energy requirements [47, 48]. The "truncated" TCA cycle therefore cannot be considered a general characteristic of the Warburg effect.

The demand for glutamine in many cancer cells far exceeds the requirement for nucleotide synthesis or maintenance of the non-essential amino acid pool, indicating that by using glutamine as an energy substrate the TCA cycle can provide proliferating cells with building blocks for proteins, nucleotides and lipids and, at the same time, stimulate mitochondrial oxidative phosphorylation via the increased amounts of reduced coenzymes delivered to the electron transport chain. The importance of glutamine as an energy substrate in cancer cells is further illustrated by the observation that various oncogenes, such as Myc, stimulate glutaminolysis through a transcriptional program [67, 68].

Contribution of the TCA cycle and oxidative phosphorylation to the energy requirements of cancer cells

Recent studies that have focused on metabolic pathways and their regulation in proliferating cells suggest that the glycolytic pathway alone can account for the ATP synthesis required to drive the biosynthetic pathways and cell survival. Aerobic glycolysis can rapidly produce ATP in sufficient amounts to drive anabolic processes in proliferating cells. However, this is true only if there is an abundant glucose source, which has important implications for the metabolic changes that occur in normal proliferating cells and cancer cells. *In vivo*, cells are frequently exposed to fluctuations in glucose and nutrient availability. In particular, cancer cells face restrictions in nutrient availability (and its subsequent metabolic implications) associated with an increase in tumor mass. As the tumor grows, the metabolic demands also increase, imposing an important metabolic challenge, i.e., how to survive fluctuations in the availability of nutrients and oxygen when tumor growth outpaces the delivery capacity of the existing vasculature.

Considering that normal cells proliferate only when stimulated by growth factors whereas tumor cells proliferate in a growth factor-independent manner, any analysis of the energy contribution of aerobic glycolysis to proliferating cells must take into account the normal or transformed nature of the cells. The specific location of a cell within the tumor mass must also be considered since this location will influence the availability of nutrients and oxygen and, consequently, the metabolic adaptations occurring within each cell. Specific metabolic adaptations can therefore occur in different populations of cells, in agreement with the metabolic heterogeneity of the tumor mass. This metabolic heterogeneity

influences cancer metabolism studies and is critical to the development of anticancer therapies.

The importance of distinguishing the metabolic adaptations that occur in normal proliferating cells from those in tumor cells becomes even more evident when the functional status of glycolysis and mitochondrial oxidative phosphorylation are correlated to the triggering of biological mechanisms (such as proliferation, senescence and apoptosis) that control the cell´s fate. During proliferation, cells must rapidly replicate their genomes while simultaneously avoiding mutations in their DNA. Normal cells have a variety of checkpoints that allow mitosis to proceed only when the genetic material is correctly replicated, thereby maintaining its intactness throughout replication. Tumor suppressor genes, such as p53, have a critical role in modulating the progression of proliferation and in inducing the metabolic changes necessary for cell survival and correct genome replication. In normal proliferating cells, the metabolic program driving cellular growth is apparently regulated by tumor suppressors in such a way that mitochondrial oxidative phosphorylation rates are controlled to avoid an increase in ROS production by high rates of electron transport chain activity. In tumor cells, which often lose important tumor suppressor genes, the situation is different. As demonstrated by Serrano et al. [69], the acquisition of tumorigenic characteristics by rodent cells transformed with oncogenic Ras is associated with the activation of p53 and p16, which is accompanied by cell cycle arrest and the appearance of a senescence-like phenotype. When p53 or p16 are inactivated, the cell cycle progresses normally. These results indicate that, in normal proliferating cells, the rates of mitochondrial respiration must be kept regulated and limited to avoid the inhibition of cell cycle progression by tumor suppressor genes; this could explain the high rates of aerobic glycolysis and lactate production in these cells. Interestingly, in cancer cells with tumor suppressor mutations, oncogenic activation can be accompanied by increased mitochondrial respiration without cell cycle inhibition and senescence. This observation suggests that energy production in normal proliferating cells is based on aerobic glycolysis, whereas cancer cells obtain a significant amount of their energy through increased rates of mitochondrial oxidative phosphorylation.

In general, any assessment of the relative contribution of glycolysis and the TCA cycle/mitochondrial respiratory chain in meeting the energy demands of proliferating cells is a challenging task. Apart from the metabolic adaptations required to cope with

proliferation, normal and tumor cells actually show different metabolic changes, indicating that these two groups of cells must be analyzed separately. In addition, the potential influence of the surrounding environment on the metabolic adaptation of cancer cells and the existence of short-term mechanisms that enable these cells to continuously alter the functional status of the TCA cycle and mitochondrial respiration must be considered. Since tumor metabolism depends on nutritional status, tumor cells can continually switch between oxidative metabolism and fermentation [62, 70].

The relative roles of aerobic glycolysis and mitochondrial oxidative phosphorylation in proliferating cells, and especially in cancer cells, are far from completely understood. However, it is clear that the metabolic adaptations associated with proliferation in cancer cells are quite distinct from those displayed by normal proliferating cells. Future advances in cancer treatment require a better knowledge of these metabolic differences.

The molecular basis of the Warburg effect

Oncogenes, tumor suppressors and metabolic adaptation

The increasing awareness of the importance of "metabolic transformation" in tumorigenesis and tumor progression has led to an improvement in our understanding of the overall metabolic changes that occur in cancer and normal proliferating cells in general and the molecular mechanisms that mediate the Warburg effect in particular.

In terms of their energy requirements and biomass production, differentiated and undifferentiated cells are quite distinct. These differences are of course highly related to the commitment of these cells to growth and proliferation or, alternatively, to the maintenance of vital processes that keep them alive and allow them to execute their biological roles. These fundamental differences in metabolic needs are reflected in the distinct regulatory mechanisms that have evolved to control cellular metabolism in proliferating and non-proliferating cells [25]. Importantly, the regulatory mechanisms that allow proliferating and non-proliferating cells to adapt their metabolism to energy and biomass production do not work in isolation. Rather, such regulation is connected to signals that reflect the overall state of the organism, with the signals being delivered to the cells through different pathways. In mammals, this

association prevents the proliferation of aberrant cells when nutrient availability exceeds the levels needed to support cell division. Indeed, cells do not normally take up nutrients from their environment unless stimulated to do so by growth factor signaling pathways.

The connection between metabolic adaptations and effective signal transduction is a critical event in cellular homeostasis and a key point in understanding processes related to the development of cancer. There is increasing evidence that many of the mutations that activate oncogenes and inhibit the activity of tumor suppressors also control the metabolic changes associated with tumorigenesis [26, 71]. Such alterations may overcome the dependence on growth factors through drastic effects on the cell´s ability to capture nutrients and in the functional status of specific metabolic pathways that promote cell survival and fuel cell growth (Fig. 3). Oncogenic mutations can stimulate the uptake of nutrients, particularly glucose, that meets or exceeds the energy demands for cell growth and proliferation [25].

The PI3K/Akt pathway: controlling the metabolic changes during cell growth

The PI3K/Akt signaling pathway, which acts downstream of various growth factor receptors and angiogenesis inducers, plays a critical role in promoting growth under normoxic and hypoxic conditions [72]. The importance of the PI3K/Akt pathway in cancer genesis and progression is demonstrated by the number of important mutations affecting its transducer molecules. The amplification of PI3K signaling, the presence of PI3K mutations and the loss of the tumor suppressor PTEN (phosphatase and tensin homolog on chromosome 10) are common in various human tumors. Genetic alterations upstream and downstream of PI3K signaling molecules, such as those affecting receptor tyrosine kinases and PKB/Akt, respectively, are also frequent in human malignancies [73].

Activation of the PI3K/Akt pathway is probably the most important event in the regulation of cell metabolism since it may drive glycolysis and lactate production, the biosynthesis of important biomolecules and the suppression of macromolecular degradation in cancer cells [22]. Additional cellular functions of PKB/Akt are related to cell cycle progression, survival and angiogenesis, the latter being a central event for tumor growth and metastasis. Special attention has been given to signaling through the kinase mTOR (mammalian target of rapamycin) which integrates signals from the PI3K/Akt
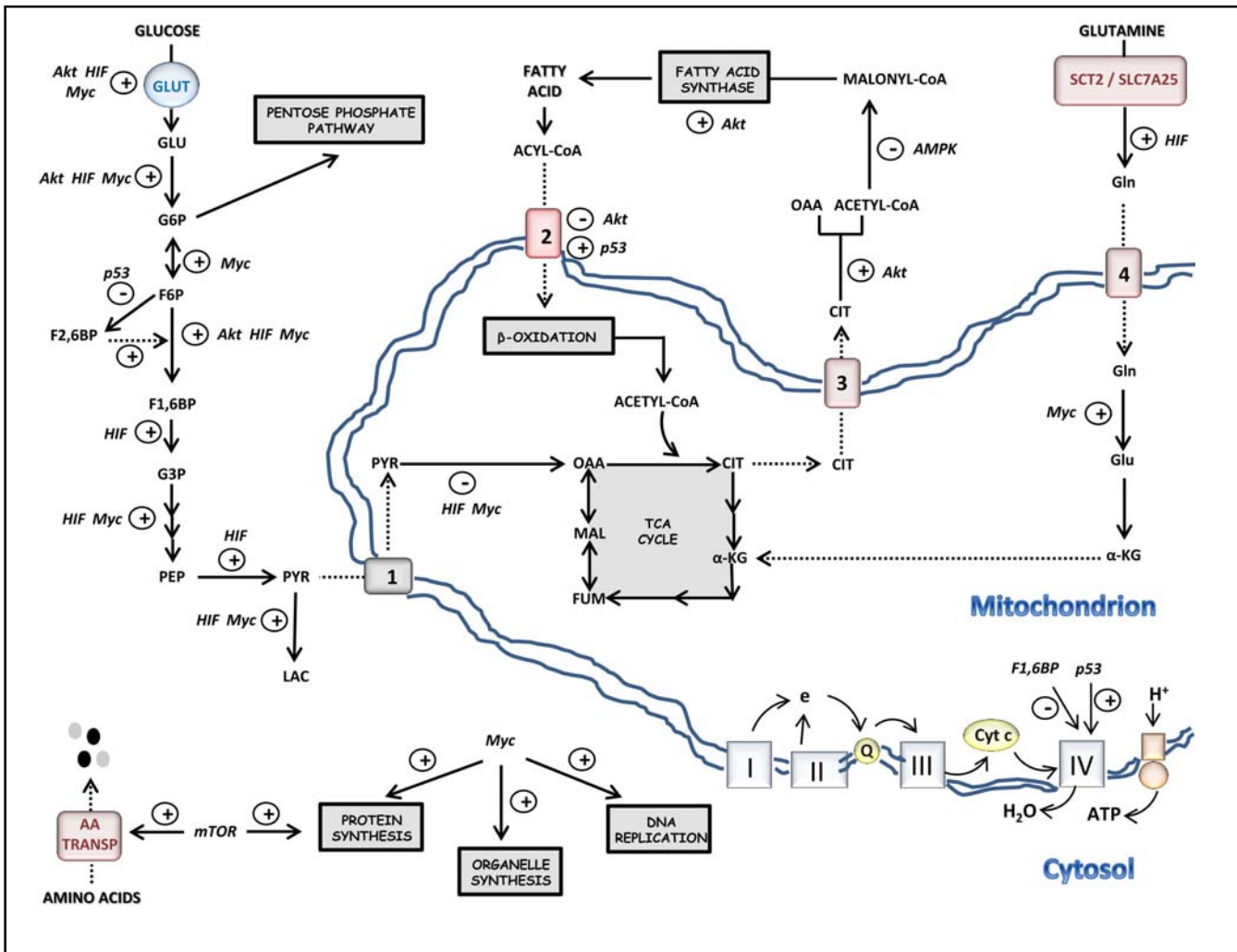
Fig. 3. Oncogenes and tumor suppressors drive the metabolic adaptation in tumor cells. Akt, mTOR, Myc, HIF and AMPK regulate the activities of metabolic pathways associated with the use of glucose, amino acids, glutamine and fatty acids in tumor cells. AA Transp: amino acid transporter; CIT: citrate; Cyt c: cytochrome c; F1,6BP: fructose 1,6-bisphosphate; F2,6BP: fructose 2,6-bisphosphate; F6P: fructose-6-phosphate; FUM: fumarate; G3P: glyceraldehyde 3-phosphate; G6P: glucose-6-phosphate; Gln: glutamine; GLU: glucose; Glu: glutamate; GLUT: glucose transporter; LAC: lactate; MAL: malate; OAA: oxaloacetate; PEP: phosphoenolpyruvate; PYR: pyruvate; Q: coenzyme Q; SCT2 and SLC7A25: glutamine transporters; α-KG: α-ketoglutarate; 1: pyruvate transporter; 2: carnitine palmitoyltransferase; 3: tricarboxylate translocase; 4: glutamine transporter.

pathway and information on the nutrient status to regulate cell growth and proliferation [72]. The activation of PI3K/Akt/mTOR signaling enhances many of the metabolic activities associated with the increase in cellular biomass in cancer cells, as described below.

PI3K/Akt/mTOR activation enhances the expression of surface nutrient transporters. Even in tissues that are not dependent on insulin, PI3K signaling through PKB/Akt can increase the expression of nutrient transporters at the cell surface, thereby enhancing the uptake of glucose, amino acids and other nutrients.

Glucose metabolism is stimulated by an increase in glucose transporters and HK expression that enhance glucose entry and metabolism [6].

PKB/Akt increases glycolysis and lactate production, as well as glutamine metabolism, through effects on gene expression and enzyme activity. PKB/Akt activation also contributes to glucose metabolism by promoting glucose phosphorylation and its retention within the cell. The PI3K/Akt pathway stimulates HK1 and HK2 activities and promotes the flux of phosphorylated glucose through the glycolytic pathway stimulated by increased

PFK activity. The activation of this signaling pathway also increases the expression of glycolytic genes [22, 25]. The glycolysis-inducing activities of PKB/Akt also contribute to apoptosis resistance in cancer cells. PKB/Akt induces the translocation of HK2 to the outer mitochondrial membrane where it binds to VDAC. This event is thought to be associated with the effects of PKB/Akt on the glycogen synthase kinase 3 (GSK3)-mediated phosphorylation of VDAC or the phosphorylation of HK2 by PKB/Akt itself [74, 75]. Once associated with VDAC, HK2 may efficiently couple residual ATP from oxidative phosphorylation to the initial and rate limiting step of glycolysis thereby stimulating the glycolytic pathway. In addition, the binding of HK2 to VDAC may inhibit mitochondrial membrane permeabilization and the consequent induction of apoptosis through formation of the permeability transition pore complex (PTPC) [21, 76].

The increased glycolysis seen in normal proliferating cells and cancer cells, even during oxygen availability, reflects the ability of PKB/Akt to activate the hypoxia-inducible factor 1 (HIF-1) transcription factor complex through upregulation and stabilization of the HIF-1α subunit, an event associated with the activation of mTOR and inhibition of the forkhead transcription factor 3a (FOXO3a). HIF-1 enhances glycolysis by increasing the expression of genes encoding glucose transporters (GLUT 1, GLUT 3), glycolytic enzymes (HK1 and 2, PFK1 and 2, aldolase A and C, GAPDH, PGK1, enolase 1, PK-M2) and LDH-A [77]. HIF-1 also regulates mitochondrial respiration by increasing the expression of the regulatory enzyme pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inactivates the PDC. This event limits the entry of glycolytic carbon into the TCA cycle and increases the conversion of pyruvate to lactate [6]. HIF-1 also induces the transcriptional activation of BNIP3, which encodes a member of the Bcl2 family that, in turn, triggers selective mitochondrial autophagy [78]. These activities indicate that HIF-1 is a critical player in the metabolic shift towards glycolysis in cancer cells.

Constitutive stabilization of the HIF-1α subunit during normoxia may occur in cancer and appears associated not only with the ability of PKB/Akt to stabilize this subunit. Under normal oxygen tension, HIF-1α accumulation is suppressed by prolyl hydroxylation, which results in ubiquitination of this protein by the von Hippel-Landau (VHL) tumor suppressor and its subsequent proteosomal degradation. ROS inhibit prolyl hydroxylase activity to promote HIF-1α stabilization, even in the presence of oxygen. This event contributes to the high rate of aerobic glycolysis in tumor cells, even in non-hypoxic conditions.

PI3K/Akt activation enhances the biosynthesis of macromolecules through changes in the expression of a variety of genes. In numerous cell types, PKB/Akt promotes the phosphorylation and activation of ACL and stimulates the expression of lipogenic genes, such as fatty acid synthase, as well as lipid synthesis in general. PKB/Akt also inhibits the oxidation of fatty acids through transcriptional downregulation of carnitine palmitoyltransferase 1A [21]. Furthermore, PI3K/Akt-mediated activation of mTOR stimulates an increase in protein biosynthesis. mTOR coordinates protein synthesis by regulating amino acid uptake, tRNA charging and initiation of translation. Signal transduction by mTOR promotes increased amino acid uptake by upregulating and maintaining the surface expression of amino acid transporters. In addition, mTOR induces the initial phase of protein translation by altering the activity of components of the translational machinery, such as eIF4F, stimulating the ribosomal S6 kinase (p70S6K) and increasing the expression of RNA polymerase III-dependent initiator methionine tRNA [26].

PI3K/Akt suppresses macromolecular degradation in cancer cells. Autophagy is a dynamic process involving the bulk degradation of cytoplasmic organelles and proteins. Autophagy is essential for the maintenance of cellular and metabolic homeostasis, mainly through the production of amino acids, ATP-generating substrates and continuous removal of either functionally redundant or aberrant intracellular structures [79]. Tor/mTOR plays a central role in regulating autophagy from yeast to mammalian cells. Through mTOR, PI3K/Akt inhibits catabolic reactions stimulated by autophagy, predominantly by activating the downstream molecule p70S6K.

The LKB1/AMPK pathway: a tumor suppressor axis linking metabolic status and cell growth

The AMP-activated protein kinase (AMPK) is a sensor of cellular energy status and is activated under stress conditions, such as hypoxia and nutrient deprivation, in which intracellular ATP levels decrease while the AMP concentration increases. In this situation, AMPK is phosphorylated by its major upstream kinase, liver kinase B1 (LKB1), and, as a consequence, cell growth is halted and ATP-consuming processes are attenuated [80]. This action is consistent with the role of LKB1 as a tumor suppressor. LKB1 gene mutations were originally described in Peutz-Jeghers syndrome, an inherited cancer disorder [81], although somatic LKB1 mutations also occur in non-small cell lung cancers

[82] and cervical carcinomas [83, 84]. LKB1 deletion is associated with hyperplasia and tumorigenesis in some tissues [85].

The mTOR pathway is a key cancer-related target of the LKB1/AMPK pathway. Under energy stress, activated AMPK can directly phosphorylate two important components of the mTOR complex 1 (mTORC1), namely, the tuberous sclerosis complex 2 (TSC2) tumor suppressor and the scaffold protein raptor. As a consequence, mTORC1 and its downstream effects on protein translation and cell growth are inhibited [80]. In contrast to PKB/Akt signaling, AMPK phosphorylates and activates the transcription factor FOXO3a and stimulates p53-induced apoptosis and mTOR inhibition; these observations indicate opposing effects of PI3K/Akt and LKB1/AMPK on cell growth and metabolism [86].

Of importance is the central role of AMPK in regulating glucose and lipid metabolism as a function of the energy and nutritional status of the cell. Activation of AMPK directly phosphorylates and inhibits acetyl-CoA carboxylase and HMG-CoA reductase, thereby reducing fatty acid and cholesterol synthesis [87]. AMPK is also associated with the downregulation of glycolysis through the phosphorylation of PFK2. The demonstration that deletion of the PFKFB3 gene (an inducible PFK2 isoform) inhibited the transformation of mouse lung fibroblasts and the tumor growth suppressing activity of PFK2 inhibitors *in vivo* [88, 89] led to the proposal that pharmacological inhibition of this enzyme may suppress glycolysis and tumor growth [90]. In addition, Shackelford et al. [91] have recently described increased levels of HIF-1α, GLUT1 and HK in LKB1- and AMPK-deficient mouse embryonic fibroblasts, and their downregulation by rapamycin. Similar results were found in patients with Peutz-Jeghers syndrome, indicating a potential role for HIF-1α as a metabolic mediator of LKB1 deficiency.

Specific mTORC1 inhibitors have been developed for clinical use, and these drugs have been anticipated to provide efficient treatment for cancer and hamartoma syndromes [92]. In this scenario, the drug metformin, an activator of AMPK commonly used to treat patients with type 2 diabetes, has been identified as a promising drug for the treatment of cancer. Indeed, studies with cancer patients presenting diabetes demonstrated that those treated with metformin were more likely to be cancer free over eight years than those on other treatment regimens [93]. Metformin is currently being tested in phase I and phase II clinical trials.

Myc family genes: metabolic adaptations to cell cycle entry and genome duplication

To proliferate, cells must achieve two main goals, namely, increase their biomass (cell growth) and duplicate their genetic material. Accordingly, proliferation requires metabolic adaptations to increase the biosynthesis of molecules such as lipids and proteins and sustain the massive nucleotide biosynthesis required for genome duplication. While the first of these demands on cellular metabolism is regulated by growth factor signaling pathways, the metabolic changes required for genome duplication depend on the activation of genes that modulate entry into the cell cycle in response to proliferative signals.

The Myc family of genes (c-Myc, L-Myc, S-Myc and N-Myc) encodes transcription factors that regulate a variety of cellular processes, including cell growth and proliferation, cell cycle progression, energy metabolism, differentiation, apoptosis and cell motility. In most human cancers, Myc activity is altered by single nucleotide polymorphisms, chromosomal translocations and gene amplification. Enhanced Myc expression is seen in 70% of all human cancers and the suppression of its expression may lead to tumor regression [94-96].

The Myc genes link altered cellular metabolism to tumorigenesis through a variety of activities that reinforce the metabolic changes induced by growth factors. Additionally, Myc exerts a key role in organelle biogenesis, which is required for energy production, biosynthesis and cell growth. Myc also stimulates entry into the cell cycle and the DNA duplication required for cellular division. Myc was recently demonstrated to have a direct role in controlling DNA replication via a transcription-independent mechanism based on its interaction with the pre-replicative complex during DNA synthesis [97]. The control of protein expression by Myc is mediated through effects on mRNA translation and the expression of a number of translation initiation factors [98].

Myc cooperates with HIF-1 to regulate genes involved in glucose and glutamine metabolism. Like the PI3K/Akt pathway, Myc is a strong inducer of enzymes associated with glucose metabolism. Most of the glycolytic and glucose transporter genes, such as LDH-A, GLUT1, HK2, PFK, hexosephosphate isomerase (HPI), GAPDH, PGK and enolase 1, are transcriptionally activated by Myc [8]. HIF-1 also directly regulates the transcription of many genes regulated by Myc, indicating the existence of functional interplay between Myc and HIF-1; this interplay could contribute to the Warburg effect, even under adequate oxygen tension [67, 99, 100].

The effects of Myc on glutaminolysis involve the upregulation of glutamine transporter genes (SCT2 and SLC7A25), which are direct targets of Myc, and an increase in glutaminase protein levels mediated by post-trancriptional regulatory mechanisms [67, 68]. Glutaminase is responsible for the conversion of glutamine to glutamate which is in turn converted to α-ketoglutarate and finally to malate in the TCA cycle. Malate is then transported out of the mitochondria to the cytoplasm, where it is converted to pyruvate by the malic enzyme, with the concomitant production of NADPH from $NADP^+$. Pyruvate is then converted to lactate by LDH-A. In addition to its role in modulating the increase in cell biomass and entry into the cell cycle, Myc also elevates the levels of intracellular NADPH, thereby supporting anabolic synthesis and contributing to the intactness of the replicating genome in proliferating cells. Recent work suggests that cancer cells select enzymatic mutations, such as those affecting Myc and isocitrate dehydrogenase 1 (IDH1), that influence cytoplasmic NADPH production during transformation [101].

Besides promoting glucose metabolism through aerobic glycolysis and stimulating the conversion of pyruvate into lactate, Myc can also stimulate pyruvate synthesis through glutaminolysis. During this process, the TCA cycle is supplied with α-ketoglutarate to yield precursors for the synthesis of biological molecules. In addition, α-ketoglutarate can be used by the TCA cycle to generate ATP and reduced coenzymes. In this way, glutamine stimulates mitochondrial respiration in tumor cells through glutaminolysis [62, 63]. Myc can also stimulate oxidative phosphorylation, in agreement with its role as an inducer of mitochondrial biogenesis and of genes related to mitochondrial function.

Myc regulates genes involved in the biogenesis of ribosomes and mitochondria. Organelle synthesis is a key function exerted by Myc since proliferating cells require an increase in the number of organelles to provide daughter cells with the machinery necessary for survival and growth. Furthermore, an increase in organelle number *per se* is advantageous for proliferation as it increases the synthesis of biomolecules and energy production.

Myc transcriptional activity is associated with enhanced mitochondrial mass and function. This event is related to the ability of Myc to upregulate genes important for mitochondrial biogenesis, mtDNA transcription and oxidative phosphorylation [102, 103]. The fact that the Myc proto-oncogene stimulates glucose uptake and glycolysis while at the same time promoting mitochondrial respiration (indirectly through an increase in mitochondrial number and directly through stimulation of oxidative phosphorylation) is consistent with an important role for ATP production by mitochondria in proliferating cells and lends further credibility to the hypothesis that the TCA cycle and oxidative phosphorylation are essential here. However, Myc also induces the expression of PDK1 and avoids pyruvate conversion to acetyl-CoA. As a consequence, pyruvate is withdrawn from oxidative mitochondrial metabolic pathways, an apparent paradox, considering the positive influence of Myc on mitochondrial proliferation and the stimulation of oxidative phosphorylation following expression of the proto-oncogene. In this context, the ability of Myc to promote glutaminolysis (see previous section) means that Myc can induce glutamine oxidation concurrently with aerobic glycolysis.

In addition to mitochondrial biogenesis, Myc is implicated in the synthesis of ribosomes and the stimulation of protein synthesis. The effects of Myc on ribosome biogenesis are related to the ability of this transcription factor to stimulate transcription by RNA polymerases I (for rRNA transcription) and III (for tRNA and small RNA transcription), in addition to RNA Pol II [67]. Moreover, Myc controls the expression of multiple components of the protein synthesis machinery, including ribosomal proteins, tRNA levels and key factors involved in translation initiation and elongation such as eIF4F subunits eIF4AI and eIF4GI [104, 105].

Myc induces cell cycle progression. Studies over the past 25 years have demonstrated the ability of Myc to suppress or stimulate the expression of various targets associated with the promotion or suppression of cell cycle progression. Among its effects, Myc can abrogate the transcription of checkpoint genes such as GADD45 and GAD153, p21 and p15, and inhibit the function of cyclin-dependent kinase (CDK) inhibitors. Additionally, Myc promotes cell cycle progression by stimulating cyclin D1, cyclin D2, cyclin E1, cyclin A2, CDK4, CDC25A and E2F2 [106]. As a result of Myc expression, G_1 is often shortened as cells enter the cell cycle, and Myc is essential for G_0/G_1 to S phase progression. Myc expression in G_1 facilitates cell entry into S, partly by activating the expression of cyclins and CDK4 [107].

The tumor suppressor gene p53
The p53 protein, encoded by the tumor suppressor gene p53, is a vital transcription factor that mediates cellular adaptation to a variety of stress conditions, including hypoxia, DNA damage and oxidative stress. Indeed, once stabilized and activated, p53 stimulates the

expression of genes that induce cell cycle arrest, senescence and apoptosis. p53 also regulates the cellular potential for angiogenesis and, importantly, can coordinate the function of metabolic pathways by triggering stress-induced transcriptional programs in order to maintain energy homeostasis. In this context, p53 mediates metabolic adaptation through activation of AMPK by energy-related stress signals. The activation of p53 by metabolic stress is associated with its phosphorylation that is directly mediated through AMPK. Once activated, p53 alters the function of specific catabolic pathways and stimulates macroautophagy, in addition to other actions. p53-induced expression of the gene TIGAR (TP53-induced glycolysis and apoptosis regulator) leads to a decrease in fructose 2,6-bisphosphate. Since fructose 2,6-bisphosphate is an allosteric activator of PFK-1, glycolysis is consequently reduced, thereby contributing to the Crabtree effect through diversion of the glucose flux to the pentose phosphate pathway and a reduction in mitochondrial oxidative phosphorylation [108]. In addition to its effects on TIGAR expression, p53 also increases mitochondrial respiration by stimulating the expression of SCO2 (synthesis of cytochrome oxidase 2), which is required for the assembly of cytochrome c oxidase [22].

The activation of p53 is associated with the stimulation of intracellular catabolic processes, including macroautophagy. Through upregulation of the damage-regulated autophagy modulator (DRAM) gene, nuclear p53 induces the expression of a lysosomal protein that stimulates the degradation of macromolecules during autophagy. The effect of p53 activation on autophagy is dualistic and dependent upon the subcellular localization of this transcription factor. Indeed, p53 activities are not limited to its action as a transcription factor and many other p53-related effects are associated with non-transcriptional actions. Thus, p53 can function as a nuclear transcription factor in the transactivation of proautophagic genes, while cytoplasmic p53 can operate in mitochondria to promote cell death and repress autophagy via poorly characterized mechanisms [109]. Finally, p53 contributes to catabolic processes by enhancing the β-oxidation of fatty acids through the action of carnitine palmitoyl-transferase, an enzyme that regulates mitochondrial fatty acid import [110].

Another important function of p53 is related to its ability to inhibit angiogenesis by inducing the expression of anti-angiogenic factors. p53 protein limits angiogenesis by at least three mechanisms: (1) interfering with central regulators of hypoxia that mediate angiogenesis, (2) inhibiting the production of pro-angiogenic factors and

(3) directly increasing the production of endogenous angiogenesis inhibitors. The combination of these effects allows p53 to efficiently shut down the angiogenic potential of cancer cells [111].

The functional status of p53 in tumor cells has always been considered an important indicator of cancer prognosis since the actions of this protein are strictly linked to the control of death and survival. The recent findings showing the consequences of p53 activation in metabolic adaptations to stress reinforce the critical role of this protein in cancer outcomes.

Metabolic therapies for cancer

Since the discovery of the Warburg effect, our knowledge of the metabolic specificities of highly proliferating normal cells and cancer cells has grown considerably. However, despite our incomplete understanding of cancer cell metabolism, several trials *in vitro* and *in vivo* have attempted to exploit our current knowledge to improve the treatment of cancer in strategies now referred to as "metabolic therapies" [10, 13]. Currently, several potential drugs that target metabolic pathways are being developed and are undergoing clinical trials [112].

The use of metabolic therapies has some advantages over other approaches. One of the driving ideas is that such therapies offer enhanced specificity since tumor cells appear to be more sensitive to metabolic inhibitors than their normal counterparts [13, 47]. Moreover, metabolic therapy may be applicable to a wide spectrum of tumor types since, regardless of the specific signaling dysfunctions, the activation of different oncogenes or loss of tumor suppressors has been associated with similar effects in tumor metabolic adaptations. Recent work has demonstrated the role of glycolysis in regulating P-gp expression, with higher glycolytic rates being related to increased P-gp expression and the emergence of the MDR phenotype in cancer cells [36]. Inhibition of the glycolytic pathway may therefore represent a potentially useful strategy for overcoming MDR in cancer therapy.

Glycolytic rates have been correlated with the degree of tumor malignancy such that faster rates are associated with de-differentiated and fast growing tumors rather than with slow growing tumors or normal cells. In addition, high glycolysis in tumor cells has been related to increased resistance to chemotherapy and radiotherapy [8]. Based on these findings, glycolysis-inhibiting drugs have been used alone or in combination with traditional

anticancer drugs to reduce tumor progression and/or enhance the efficacy of current anticancer therapies. A number of glycolysis-inhibiting drugs have successfully impaired cell growth *in vitro* [12, 113], although some of them are not very efficient in causing cell cycle arrest and/or death in certain types of cancer. A possible explanation for this is that not all tumor cells have a glycolysis-dependent metabolic program. As previously stated, many tumor cells are dependent upon the proper functioning of mitochondrial oxidative respiration, even though they have high glycolytic rates. In addition, even tumor cells that are highly dependent on glycolysis may shift between aerobic and fermentative metabolism, depending on the environmental conditions [114]. It is therefore conceivable that glycolysis-inhibiting drugs may only be useful in tumor cells that have dysfunctional mitochondrial oxidative pathways and/or oxidative phosphorylation, or those that, despite the absence of such dysfunctions, still have an absolute dependence on high rates of glycolysis. In contrast to the limitations associated with the use of glycolysis-inhibiting drugs as monotherapy, promising results have been obtained with the use of 2-deoxyglucose, 3-bromo-pyruvate, lonidamine and other compounds in combination with radiotherapy or anticancer drugs commonly used in chemotherapy. Indeed, several studies have suggested that the use of glycolysis-inhibiting drugs increases the sensitivity towards anticancer drugs [9, 115-118], and some of these drugs are being tested in phase II and III clinical trials in combination with other agents [119-123].

Drugs that inhibit oxidative phosphorylation, such as rotenone, rhodamines and oligomycin, have also been tested for their ability to impair the proliferation of tumor cells that have functionally normal mitochondria. However, two main problems have appeared with this strategy. The first problem is the apparent inefficiency of such drugs to significantly impair tumor cell growth, at least *in vitro* [124]. The ability of tumor cells to increase their glycolytic rates and reduce dependency on mitochondrial metabolic pathways may be potentiated when they are exposed to a glucose-rich environment during experiments *in vitro*; this question deserves further investigation. The second problem is the need for safer oxidative phosphorylation-inhibiting drugs than those currently available.

The results obtained with glycolysis- or oxidative phosphorylation-inhibiting drugs support the notion that tumors are highly adaptable in their metabolism and can adjust their metabolism to suit changing environmental conditions. This adaptability suggests that tumor cell metabolism cannot be suppressed by using glycolysis or oxidative phosphorylation inhibitors alone but, rather, that a combination of these compounds or their association with traditional chemotherapeutic drugs may be a potential strategy.

In addition to the use of inhibitors of glycolysis and oxidative phosphorylation, metabolic therapies have also targeted other aspects of tumor metabolism. The inhibition of glucose transport by GLUT-inhibitors has yielded positive results *in vitro* and *in vivo* [125, 126]. Although none of these drugs are currently in clinical use, GLUT transporters have been considered more adequate therapeutic targets than the direct inhibition of glycolysis [127]. In addition, given the advantages of lactate production and release to cancer cells, the suppression of membrane lactate transport between cancer cells through the inhibition of MCT proteins may be another potentially useful anticancer therapy. MCT proteins located in the plasma membrane use facilitated diffusion to mediate the symport of monocarboxylate (pyruvate, lactate, aromatic amino acids and ketone bodies) with a proton, as well as the exchange of metabolic products between cells and organs in non-pathological conditions [10]. MCT based-studies have shown that some isoforms of these transporters, such as MCT1 and MCT2, are overexpressed in cancer cells, which suggests a need for these cells to maintain low levels of intracellular lactate. Indeed, as discussed above, the function of MCT proteins in tumor cells is related to the regulation of lactate availability and pH balance inside and outside the cells. These events are important for maintaining the lactate trade and integrated metabolism established within the tumor mass and for conferring greater mobility and angiogenic potential to cancer cells [128]. Current experimental evidence indicates reduced viability and diminished invasiveness of cancer cells in which MCT expression or function is attenuated [7, 43]. Although no MCT inhibitors are currently undergoing clinical trials, the suppression of lactate production has been highlighted as a potential strategy to combat cancer cells.

Concluding remarks

Since the original pioneering studies of Otto Warburg, research on tumor cell metabolism has proven to be a promising field for the development of novel strategies to combat cancer growth, survival, invasiveness and resistance. Recent work has demonstrated the central role played by metabolic transformation in cancer genesis, survival and progression, and has highlighted the potential

use of metabolic alterations as targets for anticancer drugs. However, despite the advances in our knowledge of cancer metabolism, there are still no answers to key aspects of the metabolic alterations associated with the onset of cancer, such as the exact role of the TCA cycle in energy production in cancer cells. Defining the metabolic differences between highly proliferating cells and cancer cells is pivotal for understanding cancer metabolism and the development of more selective antitumor therapies. Detailed analysis of the functional status of metabolic pathways in tumors has revealed a variety of metabolic conditions within the tumor and the adaptability of tumor metabolism. A better understanding of this heterogeneity in cancer metabolism, coupled to the development of combined therapies that target different metabolic pathways to counteract tumor metabolic plasticity, seems to be essential for modulating cancer metabolism and improving the efficacy of current therapies.

Despite the challenges in understanding cancer cell metabolism (heterogeneity, plasticity etc.) some success has been achieved using metabolic therapies. This success is apparently related to the increased sensitivity of cancer cells to metabolic anticancer drugs and to the fact that this approach is independent of the specific signaling or epigenetic dysfunctions associated with the origin of cancer.

Alterations in cancer cell metabolism are intricately linked to the principal hallmarks of cancer. Inhibition of the processes and enzymes that participate in metabolic reprogramming may have a dramatic effect on tumors by reverting the neoplastic phenotype, stopping growth, inducing apoptosis and/or blocking angiogenesis and invasion. Clearly, understanding tumor metabolism is a challenging task but, at the same time, provides a promising target for improving traditional anticancer therapies.

Acknowledgements

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