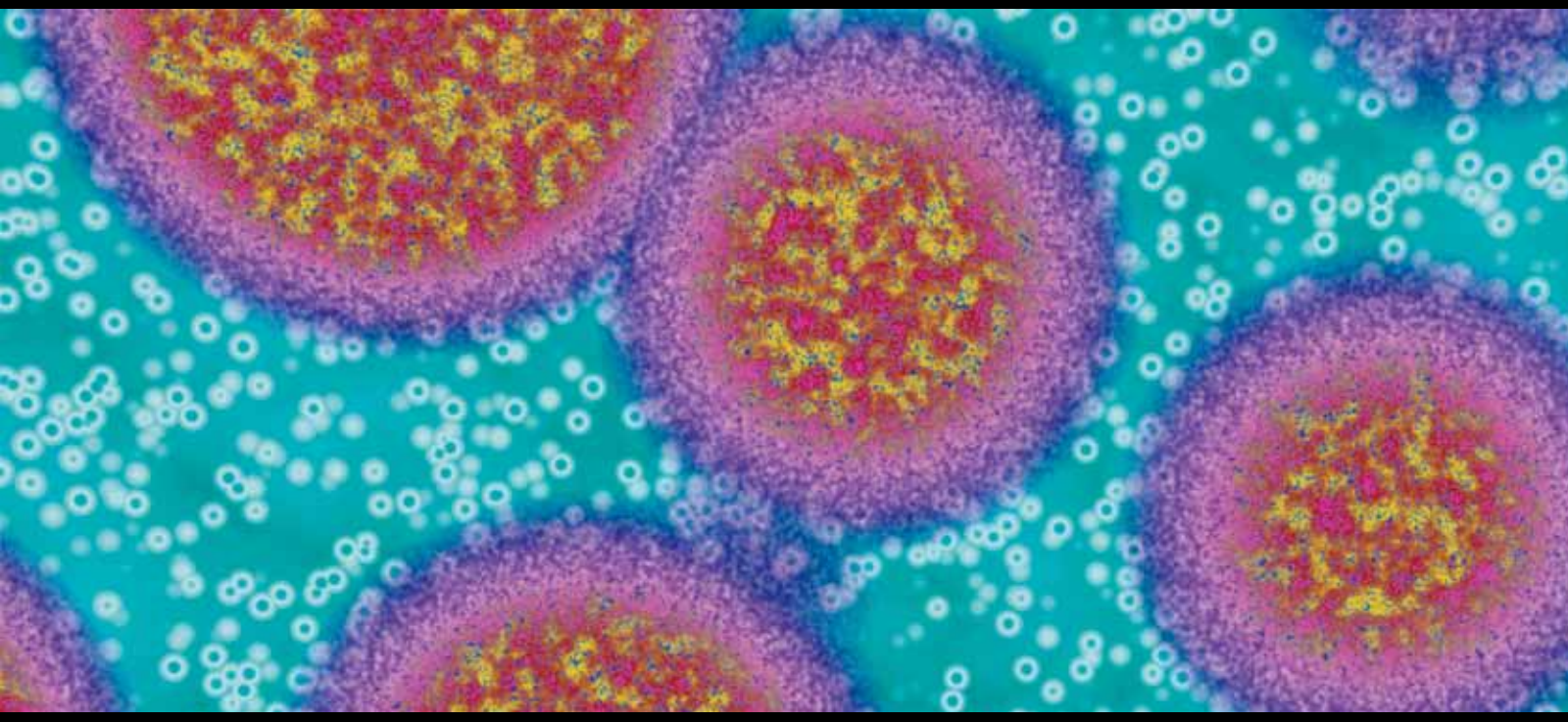
International Journal of Cell Biology

Metabolism and Cancer: Old and New Players

Guest Editors: Claudia Cerella, Carine Michiels, Roderick H. Dashwood, Young-Joon Surh, and Marc Diederich



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Editorial Board

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Editorial

Metabolism and Cancer: Old and New Players

Claudia Cerella,[1] Carine Michiels,[2] Roderick H. Dashwood,[3] Young-Joon Surh,[4] and Marc Diederich[4]

[1] *Laboratoire de Biologie Moléculaire et Cellulaire du Cancer (LBMCC), Hôpital Kirchberg, 9 rue Edward Steichen, L-2540 Luxembourg, Luxembourg*
[2] *Laboratory of Biochemistry and Cellular Biology (URBC), NAmur Research Institute for LIfe Science (NARILIS), University of Namur, 5000 Namur, Belgium*
[3] *Cancer Chemoprotection Program, Linus Pauling Institute, 307 Linus Pauling Science Center, Oregon State University, Corvallis, OR 97331, USA*
[4] *Tumor Microenvironment Global Core Research Center, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea*

Correspondence should be addressed to Claudia Cerella; claudia.cerella@lbmcc.lu

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Altered metabolism represents one of the oldest hallmarks associated with cancer. The aberrant metabolic profile deals primarily with the well-known switch of transformed cells towards aerobic glycolysis from mitochondrial oxidative phosphorylation. In addition, it deals with the exacerbation of several biosynthetic pathways interconnected with the increased glycolytic flux, eventually conferring selective metabolic advantages.

The distinctive and ubiquitous nature of this altered metabolic profile expressed by cancer cells from various origins or tissues turns the modulation/reversion of these aberrations as an amenable strategy for new anticancer therapies. Despite Otto Warburg's pioneering observations, targeting cancer cell metabolism for therapeutic purposes still remains theoretical. Several factors have contributed to this situation. Primarily, for many years, cancer has been treated as a homogeneous and too simplistic cellular system, where the impairment of proliferation/viability of the most abundant differentiated cancer cells has been extensively pursued. This approach, however, has not been successful in preventing tumour relapse. Cancer is nowadays considered a complex network, including cancer cells at different stages of differentiation, as well as noncancer cells from the tumour microenvironment. These cells exert specific functions further sustaining cancer progression, by maintaining a proinflammatory environment, by inducing activation of angiogenesis and by evading immune responses. The cellular heterogeneity implies more subtle forms of mutual interaction and communication among different cells and with the microenvironment, which may be the niche for future innovative anticancer therapeutic interventions.

In this renewed view of cancer etiology, unexpected crosstalk is emerging between metabolic mediators/processes and pathological alterations.

This special issue focuses on "Metabolism and cancer: old and new players" and intends to stimulate the discussion about new emerging targetable mediators of cancer metabolism and to evaluate possible noncanonical roles of old players in tumorigenesis as well as in cancer metabolism.

The impairment of cancer cell viability by induction of cell death remains the final outcome of many anticancer strategies. Besides apoptosis, other modes of cell death have been defined and are attracting interest as alternative therapeutically exploitable approaches to impact apoptosis-resistant forms of cancer. The review by S. Fulda, "*Alternative cell death pathways and cell metabolism*," draws attention to the programmed form of necrosis, namely, necroptosis, and discusses the relevance of metabolic pathways involved in its modulation. The author suggests a possible interconnection between alterations of redox signaling pathways mediating necroptosis and mitochondrial impairment, which implies the latter as a crucial regulator of redox imbalance.

An ideal anticancer therapy should be able to efficiently target cancer stem cells. This requires the discovery of markers selectively identifying malignant stem cells. The review by C. Pecqueur et al., "*Targeting metabolism to induce cell death in cancer cells and cancer stem cells*," addresses the relationship between cancer cell metabolism and evasion from apoptosis by comparing the main features of cancer cells and cancer stem cells. The authors correlate the peculiar metabolic characteristics of these two types of cancer cells with their ability to evade apoptosis.

Over the last 10 years, noncanonical roles for many factors implicated in apoptosis were published. The family of B-cell lymphoma-2 (Bcl-2) proteins represents one of the most interesting examples, with several members exhibiting essentially pro- and apoptotic functions. The paper by J. Michels et al., "*Functions of Bcl-xL at the interface between cell death and metabolism*," reviews new and intriguing properties of the Bcl-2 homolog Bcl-xL (B-cell lymphoma extra-large) linked to regulation of bioenergetic metabolism, which in turn controls important processes including mitochondrial ATP synthesis, Ca^{2+} flux, autophagy, mitosis, and protein acetylation.

Cancer cells rely on glycolysis rather than on oxidative phosphorylation to fulfill their energy needs. In conditions of glucose deprivation, however, cancer cells may reactivate mitochondrial bioenergetics as part of a prosurvival strategy. The study by R. Palorini et al., "*Mitochondrial complex I inhibitors and forced oxidative phosphorylation synergize in inducing cancer cell death*," shows that mitochondrial complex I inhibitors sensitize cancer cells to cell death under glucose depletion. Interestingly, combined treatments affecting glycolysis and leading to mitochondria impairment are ineffective on immortalized cells or cancer cells cultivated under high glucose conditions. Their results suggest that the forced switch from glycolysis to oxidative phosphorylation combined with the use of mitochondrial inhibitors may be used as alternative therapeutic approach to increase the sensitivity of cancer cells to death.

Identification of specific metabolic intermediates as targets for future anticancer treatments may be at the basis for attempting a reversal of tumor metabolism to normal conditions, with the rationale of impairing cancer growth. Pyruvate kinase M2 (PKM2) catalyzing the final rate-limiting reaction of glycolysis currently attracts much interest because of its multiple emerging intracellular functions. The review by N. Wong et al., "*PKM2, a central point of regulation in cancer metabolism*," gives an overview of our current knowledge about this important enzyme, discussing several of its potential contributions to tumorigenesis.

A limiting factor in exploiting cancer metabolism for therapeutic purposes is the lack of availability of agents acting as specific modulators of cancer metabolism. The review by C. Cerella et al., "*Natural compounds as regulators of the cancer cell metabolism*," covers this aspect, focusing on natural compounds as untapped potential regulators of cancer cell metabolism. The authors discuss some of the most important pathways and factors implicated in altered cancer metabolism and give an overview of compounds extracted from natural sources potentially targeting these different aberrantly regulated metabolic key intermediates.

The link between obesity, insulin resistance, and cancer is explored from different sides in three papers of this special issue, interestingly dealing all with hormonal forms of cancer. The paper by C. Brosseau et al., "*Role of insulin-like growth factor binding protein-3 in 1, 25-dihydroxyvitamin-D_3-induced breast cancer cell apoptosis*," aims at identifying the mechanisms involved in 1, 25-dihydroxyvitamin D_3-induced apoptosis in breast cancer cells. Their strategy suggests a role for insulin-like growth factor binding protein-3 (IGFBP-3) in vitamin D-induced apoptotic signaling and the involvement of impaired secretion of IGFBP-3 in acquired chemoresistance.

The review by J. S. Byun and K. Gardner, "*C-terminal binding protein: a molecular link between metabolic imbalance and epigenetic regulation in breast cancer*," introduces the reader to the concept of metabolic transduction by stressing the link between shifts in carbohydrate metabolism and alterations in epigenetic regulatory mechanisms. In particular, these authors investigate the family of transcriptional repressors called C-terminal binding proteins (CtBPs), whose function is controlled by NAD^+ levels, as an example of factors interacting and modulating the activity of different histone deacetylases (HDACs) depending on the metabolic status.

Finally, the review by J. H. Gunter et al., "*New players for advanced prostate cancer and the rationalisation of insulin-sensitising medication*," points to the roles played by metabolic disorders (especially related to insulin resistance) in favoring anticancer treatment failure and consequent higher cancer-specific mortality. The authors discuss the promising use of insulin-sensitizing drugs as potential anticancer agents to be used in combinational therapies.

Although alterations in cellular metabolism are an old acquaintance in cancer etiology, we still need to do more before we can translate our knowledge into new and specific anticancer therapies. This final outcome will mostly depend on an in-depth identification of the mutual modulation between metabolism processes and factors that regulate proliferation and death/survival of cancer cells. With this special issue, we hope to offer new and relevant insights towards a critical reevaluation of this rapidly moving field.

Claudia Cerella
Carine Michiels
Roderick H. Dashwood
Young-Joon Surh
Marc Diederich

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Review Article

C-Terminal Binding Protein: A Molecular Link between Metabolic Imbalance and Epigenetic Regulation in Breast Cancer

Jung S. Byun and Kevin Gardner

Genetics Branch, Centers for Cancer Research, National Cancer Institute, 41 Library Drive, Bethesda, MD 20886, USA

Correspondence should be addressed to Kevin Gardner; gardnerk@mail.nih.gov

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The prevalence of obesity has given rise to significant global concerns as numerous population-based studies demonstrate an incontrovertible association between obesity and breast cancer. Mechanisms proposed to account for this linkage include exaggerated levels of carbohydrate substrates, elevated levels of circulating mitogenic hormones, and inflammatory cytokines that impinge on epithelial programming in many tissues. Moreover, recently many scientists have rediscovered the observation, first described by Otto Warburg nearly a century ago, that most cancer cells undergo a dramatic metabolic shift in energy utilization and expenditure that fuels and supports the cellular expansion associated with malignant proliferation. This shift in substrate oxidation comes at the cost of sharp changes in the levels of the high energy intermediate, nicotinamide adenine dinucleotide (NADH). In this review, we discuss a novel example of how shifts in the concentration and flux of substrates metabolized and generated during carbohydrate metabolism represent components of a signaling network that can influence epigenetic regulatory events in the nucleus. We refer to this regulatory process as "metabolic transduction" and describe how the C-terminal binding protein (CtBP) family of NADH-dependent nuclear regulators represents a primary example of how cellular metabolic status can influence epigenetic control of cellular function and fate.

1. Introduction

The first written description of breast cancer was recorded in 3000 B.C. as an inscription in the Smith Papyrus that pictured ulcerating lesions of the breast, a condition for which there was no cure [1]. Though, as early as Hippocrates (460–375 BC), the general belief was that cancer initiated from natural causes, a fuller understanding of cancer did not emerge until the late nineteenth century where the development of higher resolution microscopy made the visualization of cells and tissue possible [2]. This event marked the birth of modern pathology and revealed that there are striking differences in the appearance of cancer cells when compared to the surrounding normal tissue. This difference or "otherness" of malignancy made it clear that cancer develops from a change or transformation of normal tissue, a difference that modern molecular biology reveals to be rooted in genomic "changes" or mutation to cellular DNA sequence [3]. It is now widely recognized that mammalian cells are constantly exposed to genotoxic stress from both endogenous and exogenous sources that threaten to change or mutate the human genome and thereby increase the risk of cancer. To address this threat, mammalian cells and tissues have evolved numerous mechanisms and pathways to identify and repair damage to the human genome [4]. These DNA repair pathways and the manner in which cells and tissues provide surveillance to identify and remove cells that have irretrievably lost their genomic fidelity constitute what is known as the DNA damage response (DDR) [3, 4], which is now recognized as a major criteria through which clinicopathological assessment and classification of breast cancer are defined [5–8].

2. The Molecular Stratification of Breast Cancer

A significant development in the clinicopathological assessment of breast cancer has been the recognition that breast

cancer is a heterogeneous disease that can be stratified into relatively distinct entities or subtypes based on specific molecular parameters [6, 9, 10]. At least 5 different classes or "subtypes" are described, including luminal A, luminal B, human epidermal growth factor 2 (HER2) positive, basal-like, and claudin low [10]. Each of these subtypes has been shown to resemble or reflect distinct stages of mammary differentiation where the claudin low and basal-like represent the more primitive, receptor deficient, "triple negative" (lacking estrogen, progesterone, and HER2 receptors) spectrum of differentiation [11]. Most notably, a similar functional stratification exists with respect to DNA repair, where the less differentiated subtypes exhibit the greatest deficiencies in genome stability [5]. Since tumors with higher levels of genome instability typically show more aggressive behavior, it is clear that this stratification of breast cancer phenotypes and its functional correlation with DNA repair capacity have significant prognostic importance that will guide therapeutic strategies [6, 7, 12, 13]. These relationships are well demonstrated by the observation that the gene products of the early onset breast cancer genes BRCA1 [13, 14] and BRCA2 [15], whose germline loss or mutation confers a near 80% risk of developing breast cancer, are themselves DNA repair proteins [16]. Moreover, patients with germline depletion of BRCA1 give rise to tumors of the more primitive, triple negative, basal-like, and claudin-low subtypes [17, 18]. Similarly, sporadic tumors that show deficiencies in BRCA1 tend to be of the basal-like or claudin-low subtype, and estimates indicate that as many as 40% of nonhereditary or sporadic breast cancers show decreased expression of BRCA1 [19].

3. Obesity, Diabetes, and the Risk of Breast Cancer

It is estimated that obesity in United States accounts for nearly 15%–20% of cancer deaths [20, 21]. Cancer death rates from women who are obese are from 50% to 60% higher than women who are of normal weight. Breast cancer rates in postmenopausal women increase 30%–50% with obesity and are associated with more aggressive disease. In fact, very obese women with a body mass index (BMI) greater than 40.0 kg/m^2 have a three times higher risk of death from disease compared to their much leaner (BMI < 20.5 kg/m^2) counterparts [22]. Finally, as might be predicted, patients who are both obese and have germline BRCA1/2 mutations show significantly increased risk over either condition alone [23].

The relationship between obesity and breast cancer is complex. Most studies have focused on the abnormally elevated levels of circulating mitogenic hormones and inflammatory cytokines [22, 24, 25]. The rise in adipocyte size due to calorie excess causes increased release of free fatty acids and enhanced secretion of peptide hormones such as leptin, resistin, and tumor necrosis factor alpha (TNFα) and reduced release of adiponectin. Adipose cells also express significant levels of steroid hormone metabolizing enzymes. The high levels of TNFα and reduced level of adiponectin give rise to insulin resistance and type II diabetes [24, 25]. The elevated insulin levels result in increased production of insulin-like growth factor one (IGF-1), both of which promote cellular proliferation. Increased levels of steroid hormone metabolizing enzymes like aromatase in adipose cells convert circulating androgen precursor to estrogens. This rise in free circulating estrogen is further exacerbated by the reduced synthesis of the sex-hormone binding globulin (SHBG) due to the obesity-associated hyperinsulinemia [24–26].

4. The Molecular Cost of Metabolic Imbalance

To define the link between obesity and cancer we must begin by understanding the molecular impact or "cost" of metabolic imbalance. Conditions of excess calories or "overnutrition" have profound effects on cellular metabolism [26]. Oxidation of free fatty acids, glucose, and other carbon intermediates by beta-oxidation, glycolysis, and the tricarboxylic acid cycle transfers electrons primarily to nicotinamide adenine dinucleotide (NAD+) to produce NADH. The NADH produced in this fashion normally has several different fates including its oxidation in the presence of molecular oxygen via the mitochondrial electron transport chain to produce H_2O and ATP. One well-understood consequence of the elevated NADH levels due to nutrient excess is the increased generation of reactive oxygen species (ROS) like superoxide (O_2^-) as a consequence of incomplete mitochondrial electron transfer during respiration [27, 28]. The ROS, thus generated, contributes significantly to the risk of malignant transformation by causing DNA damage.

5. NAD+/NADH and Cancer Metabolism

The NAD+/NADH redox imbalance of nutrient excess and obesity is reminiscent of the metabolic imbalance associated with the malignant proliferation of cancer, first described by Otto Warburg in 1927 [26, 103–111]. In his work, Warburg noted that cancer cells demonstrated high levels of glucose consumption and lactic acid production even though there was significant oxygen to sustain the respiratory production of energy by the oxidation of NADH via oxidative phosphorylation. The net result is an elevation in the steady state levels of cellular NADH. This shift in energetic carbon flux is referred to as the *Warburg Effect* and has been the focus of extensive investigation as a potential vulnerability of cancer metabolism ("cancer's sweet tooth") that may be exploited for chemotherapeutic benefit [106, 107, 109, 111].

In addition to threats to genome integrity from elevated ROS, the shifts in the redox status and availability of NAD+, due to either calorie excess or the *Warburg Effect*, have profound influence on certain specialized classes of mammalian proteins that directly utilize oxidized or reduced NAD+ as cofactors, ligands, or substrates. Some of these factors have widespread impact on genome integrity by controlling the DNA damage response and include the Sirtuin family of Class III histone deacetylases [112, 113], the PARP family of poly ADP ribosyl-transferases [114–116], and the C-terminal binding protein (CtBP) class of transcriptional

repressors [117–120]. The relative K_M of the Sirtuin and PARP family are in the 50–200 micromolar range [121]. Since the levels of free cytoplasmic NAD+ are in the range of 500–800 micromolar, both the Sirtuin and PARP families are functioning at saturation. In contrast, the free cytoplasmic concentration of NADH is 1 micromolar, while the CtBP binding protein (CtBP) class of transcriptional repressors has binding affinity in the 100 nanomolar range [122]. Therefore, CtBP is likely to function as a true sensor of cellular metabolic status by sensing acute and chronic changes in cytoplasmic and nuclear levels of NADH.

This review will summarize the role of CtBP in the maintenance of genomic homeostasis and describe how its activity links cellular metabolic status with genome stability and epithelial reprogramming in breast cancer and how this linkage has broader implications for other epithelial cancers.

6. C-Terminal Binding Protein Structure and Function

CtBP is expressed from two genes, *CtBP1* and *CtBP2*. Both of these genes give rise to many different isoforms, some displaying distinct functions. There are essentially four major CtBP isoforms: *CtBP1-L*, a shorter isoform of *CtBP1* (CtBP1-S) referred to as *CtBP3/BARS*, CtBP2 (including a *CtBP2-L* and shorter splice isoform *CtBP2-S*), and *RIBEYE*, a variant that contains a large N-terminal domain and is transcribed from an alternate *CtBP2* promoter [119]. CtBP was first identified as a phosphoprotein that interacted with the C-terminal protein sequences encoded in exon 2 of the oncogenic adenovirus 2/5 E1A protein [123]. The CtBP protein was later found to function as a transcriptional corepressor when targeted to gene promoters through the C-terminal sequences of E1A [124]. This binding was mediated by a highly conserved 5-6 amino acids binding motif (PXDLSK) that adopted the conformation of a series of beta turns in solution [125]. The PXDLS binding motif was later found in a variety of transcriptional repressors across different species, including drosophila transcriptional regulators that play broad roles in tissue morphogenesis, like zfh-1, hairy, knirps, giant, kruppel and snail and its mammalian homologues snail, and ZEB1/2 [29–32, 126]. A search for other proteins that interact with CtBP led to the identification of C-terminal binding protein interacting protein (CtIP) [127], a protein that was later implicated in having a significant role in maintaining genome stability through its interaction with the early onset breast cancer gene, *BRCA1* [35], indicating that CtBP could bind to a diverse array of factors with distinct and overlapping molecular functions. This link was later expanded when it was found that Rb pocket binding LXCXE motif within CtIP enabled it to form higher order complexes with CtBP and Rb family members via a separate domain containing the common PXDLS motif indicating that CtIP/CtBP complexes represent a corepressor assembly that could recruit tumor suppressors like Rb in the context of histone deacetylase activity. Subsequently, CtBP began to show up in a variety of other protein interaction screens as binding partners for several different transcriptional regulators that control diverse cellular programs including Net (*ELK4*), a member of the ternary complex family involved in regulation of *Fos* and other immediate early gene expressions through the serum response element, and the transcriptional repressor KLF8 (*ZNF741*) [36, 37].

7. Early Clues of Role for CtBP in Development

CtBP is well conserved, and forms of CtBP are expressed from men to flies, worms, and plants [118, 128, 129]. Consistent with its role in tissue morphogenesis, first demonstrated in drosophila, several studies have identified a role for CtBP in driving mammalian epithelial programming where the cellular adhesion molecule E-cadherin is a major target of repression through the recruitment of CtBP/ZEB complexes to multiple E-box transcription factor binding sites (TFBS) in the E-cadherin (*CHD1*) promoter [130]. This study also shows that CtBP inhibits cell anoikis, suggesting a prominent role for CtBP in promoting the early stages of epithelial-to-mesenchymal transition [130, 131]. Other lines of evidence are beginning to suggest significant roles for CtBP in epithelial reprogramming. For instance, gene expression studies in CtBP depleted cells reveal that multiple epithelial and proapoptotic gene pathways are regulated by CtBP [38]. Subsequently, several other genes have been found to be transcriptionally regulated by CtBP, including the telomerase protein and RNA components, *TERT* and *hTERC* [132], the notch target gene *Hey1* [74], and the brain-derived neurotrophic factor (*BDNF*) promoter through binding the REST transcription factor [133]. Transforming growth factor beta (TGF-β) plays a ubiquitous and multipotent role in regulating tissue morphogenesis. Under certain conditions, its influence is antiproliferative; yet under others, it can promote tumor progression and invasion [134]. The CtBP interaction with the transcriptional regulator Evi-1 protein, known for inducing blocks to differentiation that promote leukemogenesis, is also known to inhibit signaling through TGF-β by associating with Smad3 containing complexes and recruiting CtBP [39]. This interference with TGF-β signaling is also facilitated through the interaction of CtBP with inhibitory Smad 6 [60]. Accordingly, Evi-1 induced transformation of Rat1a fibroblast requires CtBP, the first indication that CtBP plays a direct role in cellular transformation [135]. Similarly, overexpressed and t(3;21) chimeric fusions of Evi-1 also function through CtBP to block differentiation and promote leukemogenesis [136, 137]. CtBP transgenic studies show that CtBP plays roles in a vast variety of developmental functions [138]. While CtBP2 deletions are an embryonic lethal (E10.5), mice with CtBP1 disruption are viable and fertile but die early [118, 138].

8. CtBP and NADH

It was not until nearly a decade after its first discovery that CtBP was found to be an NADH regulated dehydrogenase of the well-conserved D2 hydroxyacid dehydrogenase class that undergoes a conformational change in association with either NAD+ or NADH [139]. Moreover, it was found that

residues associated with the active site are linked to the ability of the dimeric components to bind to the PXDLS peptide motif on its binding partners [139]. Like other D2 hydroxyacid dehydrogenases (e.g., GAPDH), CtBP was found to form higher order oligomers and increase its interaction with PXDLS containing protein domains in the presence of NADH and NAD+ [140]. Moreover, its interaction with PXDLS containing peptides slowed the catalytic activity [140]. Notably, the binding affinity of CtBP was over 100-fold higher for NADH than NAD+ suggesting a substantial role for CtBP as a metabolic sensor of redox status [122, 141]. However, ablation of enzymatic activity by mutations at histidine-315 showed no effect on CtBP transcriptional regulation, suggesting that NADH/NAD+ binding, not the dehydrogenase activity of CtBP, was necessary for its repressor activity [38, 142]. Nonetheless, a developmental study in drosophila revealed distinct phenotypes for CtBP with impaired enzymatic activity; so, the identity and role of the true substrate for the CtBP dehydrogenase remain a mystery [143].

9. CtBP and Regulation of the Epigenome

Several reports demonstrate that CtBP forms complexes with a variety of epigenetic regulators or corepressor complexes that recruit epigenetic regulators. Initial studies show that CtBP interacts with consensus binding motifs on class II histone deacetylases (HDAC 4, 5, and 7) and the corepressor protein MEF2-interacting transcription repressor (MITR/HDAC9), a structural scaffold that associates with other HDACs, through amino terminal sequences [40]. The class I histone deacetylases (HDACs, 1, 2, and 3) are also found to associate with CtBP through various multicomponent complexes [42, 144]. In multiple studies, these complexes were found also to contain several different types of epigenetic regulators, including histone methyltransferases G9a and EHMT1; the G9a and EHMT1 binding zinc finger protein, WIZ, that bridges interaction with CtBP; the histone demethylase LSD1; an actin-related component of the SWI/SNF complex, ArpNα; CoRest (RCOR1), a corepressor protein that interacts with REST transcription factor; CDYL, a component of the polycomb regulatory complex 2 (PRC2) that bridges interaction between repressive Histone H3K27Me3 modifications and the EZH2 histone H3K27 methyltransferase; and a component of the polycomb regulatory complex I (PRC1), CBX4 [42–44, 46]. Since both G9a and CBX4 interact with DNA methyl-transferases [145–148], it is likely that some CtBP complexes will also contain DNA methyl-transferases. Recent findings indicate that the histone acetyl-transferase, p300, also forms complexes with CtBP. In the CtBP:p300 complex, CtBP interaction with the p300 bromodomain represses p300 HAT activity [45]. The BCL6 corepressor BCOR-L1 associates with CtBP in combination with class II HDACs (HDAC 4, 5, and 7) to repress target genes like E-cadherin [47]. The estrogen receptor corepressor, Rip140, forms a complex with CtBP to participate in control of hormone regulated genes [48]. Transcriptional repression through GATA2 and GATA3 is mediated by combined association of the corepressor, friend of GATA (FOG) with CtBP to block adipogenesis [49]. Another pathway through which CtBP controls adipocyte growth and differentiation is through the transcription factor PRDM16 that recruits CtBP to shut down genes that promote white adipose cell growth and differentiation and eventually exchanges CtBP factors for PGC-1α and PPARγ to drive the expression of brown fat genes [50]. Finally, it has been shown that the p53 gene product and regulator hmd2 can act as a corepressor at p53 regulated genes to recruit CtBP to mediate transcriptional repression [51].

Other DNA binding transcription factors regulated by CtBP include hypermethylated in cancer (HIC1), which forms a complex with CtBP that regulates SIRT expression; ZNF36, which is involved in the regulation of estrogen controlled genes; BCL6, which binds directly to CtBP to autoregulate its own transcription; the corepressor BCL3 whose association with DNA bound NF-kappa B dimers requires CtBP for transcriptional repression; Ikaros, a transcription factor necessary for lymphoid development, whose repressive activity requires a direct interaction with CtBP through an N-terminal PXDLS motifs and an interaction between CtBP and Sin3A; the TCF-4 of Wnt signaling whose physiological repression of CtBP target genes is lost in cancers that are deficient in mismatch repair and express TCF-4 isoforms incapable of binding CtBP [53–56, 58, 149–151].

10. Posttranslational Regulation of CtBP

CtBP protein undergoes dynamic posttranslational regulation (see Table 2). The changes influence either the stability or the subcellular localization of CtBP. CtBP2 and CtBP1 readily hetero- and homodimerize; however, only CtBP2 has a nuclear localization signal that allows it to translocate to the nucleus. Thus, CtBP1 must either enter the nucleus as a heterodimer with CtBP2 or through the formation of a complex with BKLF (KLF3) or other factors [57]. This mode of translocation is heavily dependent on CtBP1 dimerization so decreases in dimerization; through decreased availability of NADH can result in retention of CtBP1 in the cytoplasm [57]. The nuclear splicing factor Pnn/Drs has recently been found to be recruited to gene promoters by CtBP to influence splicing [152]. This interacting Pnn/CtBP complex also plays a role in sequestering CtBP in nuclear speckles to relieve transcriptional repression of CtBP-targeted genes [59]. Finally, the subcellular localization of CtBP is dynamically controlled by posttranslational modifications. Sumoylation of CtBP1 at lysine K428 results in increased nuclear retention [98]. Similarly, acetylation of CtBP2 by p300/CBP on lysine residues K6, K8, and especially K10 results in nuclear retention [153]. Interestingly, sumoylation of CtBP1 was inhibited in the cytoplasm by the PDZ protein nNOS resulting in greater cytoplasmic retention [78, 98]. The total levels of CtBP1 sumoylation appear low, and so the relative contribution of this modification to CtBP regulation remains unclear; however, CBX4 (see earlier), PIAS1, PIASxα, and PIASxβ have been shown to be the likely E3 ligases involved [154–156]. The role of CBX4 in the regulation of CtBP1 remains complicated as CBX4 promotes complex formation between

CtBP1 and ATK1, where ATK1 dependent phosphorylation of CtBP1 results in decreased dimerization and increased ubiquitination with subsequent proteasomal degradation [96]. Interestingly, CtBP1 is also phosphorylated on Ser-158 by AMP Kinase (AMPK) which results in decreased repressive function, suggesting a novel mechanism through which CtBP activities are controlled by nutrient stress [93].

Two previous reports have shown that the *adenomatous polyposis coli* gene (APC) interacts with CtBP and regulates its degradation through a proteasomal pathway [61–63, 85]. In fact, degradation or loss of CtBP was proposed as a necessary step in the evolution of colonic adenomas in a zebrafish model [63]. Another tumor suppressor gene that is associated with CtBP degradation is alternative read frame tumor suppressor gene ARF whose association with CtBP leads to its degradation [157]. Phosphorylation of CtBP on Ser-422 by homeodomain interaction protein kinase 2 (HIPK2) leads to its ubiquitination and proteasomal degradation in response to UV irradiation [65]. Similarly, c-jun NH2 terminal kinase 1 (JNK1) also phosphorylates CtBP on Ser-422 [66] suggesting that the induction of stress pathways may be a common mechanism to reduce the level of CtBP posttranslationally. Phosphorylation of CtBP on serine 158 by the p21 activated kinase PAK1 triggers relocalization to the cytoplasm [97]. Recent studies indicate that phosphorylation of CtBP on T144 by cyclic-AMP dependent kinase (PKA) leads to increased dimerization [92]. The interaction of CtBP with X-linked inhibitor of Apoptosis (XIAP) also leads to its ubiquitination and degradation. Though the mechanism of degradation has yet to be fully described, the removal of phosphorylated CtBP from promoter bound locations is thought to require the action of the transducin beta family of chaperone-like molecules, TBL1 [67]. Finally, very recently the C-terminus of Hsc70-interacting protein was found to interact with CtBP2 leading to its ubiquitination and subsequent degradation in the proteasome [95].

11. Moonlighting Functions of CtBP in the Cytoplasm

The fission and fusion of biological membranes during intracellular trafficking of membrane bound vesicles and structures support broad programs of endocytosis and exocytosis as essential cellular functions. Both forms of CtBP1 (CtBP1-L and the CtBP3/BARS/CtBP1-S) have cytoplasmic functions that remain to be clearly defined but appear to be linked to Golgi membrane fission and homeostasis [119, 158]. CtBP3/BARS is reported to have an essential role in regulating membrane fission in the Golgi tubular network and has also been implicated in mitotic partitioning of the Golgi apparatus [159, 160]. Though the mechanism and putative enzymatic activity exerted by CtBP in this processes remain unresolved [161, 162], there is general agreement for a central role of CtBP in the formation of vesicular and tubular membrane carriers that ferry membrane bound components to different intracellular compartments [163]. How this function may impact on the nuclear function of CtBP and how such activity influences the role of CtBP in development and oncogenesis remain unclear. Some reports suggest that loss of CtBP3/BARS is associated with decreased surface expression of the FAS/CD95 [164]; therefore, this mechanism may have a role in cellular survival strategies. However, given the known role of CtBP in influencing cellular reprograming and antagonizing the epithelial phenotype [118], it is tempting to speculate that this property of CtBP could have a role in defining epithelial polarity [165–167]. The partitioning of the Golgi during mitosis may also have a significant role in promoting formation and orientation of the mitotic spindle and thus could influence the asymmetric division necessary for the formation of stratified epithelia and the maintenance of pluripotent stem cell pools [168, 169]. Similar membrane trafficking promoted by CtBP3/BARS may have a role in maintaining the basolateral and apical polarity of epithelial cells during tissue morphogenesis, regeneration, and wound healing. All of these events are disrupted and deregulated in cancer. Finally, it is tempting to speculate that secretory tissues that must undergo cyclic proliferation and involution or repair (e.g., breast, endometrium, and colon) may be particularly dependent on both the nuclear and cytoplasmic functions of CtBP.

12. CtBP and Oncogenesis

Since its discovery in 1993, many studies provide evidence that CtBP plays an expanded role in the evolution and progression of cancer controlling gene expression through a variety of transcriptional regulators and gene networks (Table 1). Many of these networks are associated with malignant behavior in a variety of cell types. CtBP recruitment and transcriptional targeting of multiple genes important in hematopoietic differentiation, including *Evi-1*, *BCL3*, *BCL6*, *GATA1*, *GATA2/3*, FOG1, and Ikaros strongly implicate prominent roles for CtBP in the incidence and progression of erythroid, lymphoid, and myeloid malignancies [39, 49, 54, 56, 58, 136, 170, 171]. Similarly, the interaction of CtBP with a variety of developmentally regulated genes that control various processes in tissue development, like EMT, strongly implicates a significant role for CtBP in the incidence, growth, and progression of epithelial cancers [38, 130, 172]. As mentioned previously, the control of CtBP protein levels by APC was one of the earliest indications of a link between CtBP and epithelial cancers [62, 85, 173]. In fact, the earlier studies that linked CtBP function to EMT were carried out in lung and colonic cell lines and tissues [174, 175]. These have been augmented by studies with patient tissue showing correlation between CtBP expression and malignancy. Nadauld et al. showed that CtBP could be linked to impaired differentiation in colon cancer because it decreased the production of retinoic acid through repression of retinol dehydrogenase [62]. In this study, the investigators were able to show that, in colonic biopsies from patients with familial adenomatous polyposis (FAP) (germline deficiencies in APC, which induces CtBP degradation), adenomas showed elevated levels of CtBP1 that was correlated with reduced levels of retinol dehydrogenase expression [62]. Deng et al. found that CtBP1 expression was elevated in a large percentage patient melanomas [176].

Table 1: CtBP interacting protein complexes.

Factor	Function	Ref
Zfh-1	Transcription repression	[29]
Hairy	Transcription repression	[30]
Knirps	Transcription repression	[31]
Giant	Transcription repression	[32]
Kruppel	Transcription repression	[32]
Snail	Transcription repression	[32]
ZEB1/2	Transcription repression	[33]
CtIP	Genome stability	[34]
BRCA1	Transcription repression	[35]
NET (ELK4)	Transcription repression	[36]
KLF8 (ZNF741)	Transcription repression	[37, 38]
Evi-1	Inducing blocks to differentiation	[39]
HDAC 4, 5, 7	Histone deacetylation	[40]
HDAC 1, 2, 3	Histone deacetylation	[41, 42]
G9a	Histone methyltransferase	[42]
EHMT1	Histone methyltransferase	[43]
WIZ	Transcription repression	[44]
Lsd1	Histone demethylase	[42]
ArpNα	Transcription repression	[45]
CoRest (RCOR1)	Transcription repression	[42]
CDYL	Transcription repression	[46]
CBX4	Transcription repression	[46]
P300	HAT inhibition	[47]
BCOR-L1	Transcription repression	[48]
RIP140	Hormone regulation	[49]
FOG	Transcription repression	[50]
PRDM16	Transcription repression	[51]
Hmd2	Transcription repression	[52]
HIC1	Regulate SIRT expression	[53, 54]
ZNF366	Estrogen control gene regulation	[55]
BCL3	Transcription repression	[56]
BCL6	Autoregulation of transcription	[56]
Ikaros	Transcription repression	[57]
Sin3A	Transcription repression	[57]
TCF4	Transcription repression	[58]
BKLF (KLF3)	Stability of subcellular localization	[59]
Pnn	Transcription Repression	[60]
APC	Degradation	[61–64]
ARF	Degradation	[65]
HIPK2	Ubiquitination, proteasomal degradation	[66]
JNK1	Phosphorylation	[67]
TBL1	Dephosphorylation	[67]
CDK7/CCNH	Post-translational stability	[68]
Huntingtin	Unknown	[69]
Glis2	Transcriptional repression	[70]
PLD1	Activation of macropinocytosis	[71]
Smad6	Transcriptional repression	[60]
Ataxin	CtBP antagonism	
PARP1	Corepressor complex	[72]
Sox6	Transcriptional repression	[73]
Spen	Transcriptional repression	[74]
BCoRL1	BCL6 transcriptional Co-repression	[47]
Eos (IKaros family member)	Transcriptional repression	[75]
Acetylcholinesterase-S (AChE-S)	Antagonize CtBP transcriptional repression	[76]
SatB1	Co-repressor complex	[77]
nNos	Cytoplasmic localization	[78]
Tel/EVT6	Control of endothelial sprouting	[79]
ER-beta	Suppression of inflammatory response in CNS microglia and astrocytes	[80]
KLF12	Transcriptional repression	[81]
MLL	Transcriptional repression	[82]
HDGF	Transcriptional repression	[83]
KCNIP3/KCHIP	Calcium-dependent Transcriptional repression	[84]
MITR	Transcriptional repression	[40]

Increased expression of both CtBP1 and CtBP2 has been seen in tumors from patients with head and neck squamous cell cancers [177, 178]. A very recent study reported an elevation of CtBP2 in ovarian cancer and suggested that CtBP2 expression could be used as a marker for patients that are more likely to respond to epigenetic therapy utilizing histone deacetylase inhibitors [179]. Another group at the University of Michigan recently reported that CtBP1 was overexpressed and mislocated in metastatic prostate cancer and suggested a prominent role for CtBP1 in the progression of prostate cancer [180]. Several recent studies provide strong evidence indicating that elevated CtBP expression and activity may play a significant role in human breast cancer [172, 181–185]. In each study, increased CtBP expression was found in malignant as opposed to nontransformed patient samples. Yet, a systematic profiling of the network of genes controlled by CtBP in human breast cancer and the implication of that control in breast cancer evolution and outcome has been lacking.

Table 2: CtBP regulators

Protein	Mode of regulation	Ref.
APC	Protein degradation	[61–63, 85]
	Proteasome dependent	
HIPK2	Phosphorylation-dependent	[86, 87]
	Protein degradation	
TBL1	Protein degradation	[88]
	Proteasome-dependent	
Ink4a/Arf	Protein degradation	[89, 90]
	Proteasome mediated	[64]
BCL3	Protein Stabilization	[91]
PKA	CtBP dimerization	[92]
AMPK1	Phosphorylation dependent inactivation	[93]
XIAP	Polyubiquitination degradation	[94]
Stub1/CHIP	CtBP2 polyubiquitination degradation	[95]
AKT1	Phosphorylation induced decreased dimerization	[96]
JNK1	Phosphorylation dependent	[66]
	Proteasomal degradation	
PAK1	Phosphorylation dependent	[97]
	Translocation to the cytoplasm	
CBX4/UBC9	Sumoylation-dependent nuclear retention	[98–101]
p300	Acetylation increased nuclear retention	[102]

13. Genome-Wide Profiling of CtBP Interactions across the Breast Cancer Genome

Beginning with the observation that CtBP could act as a metabolic sensor to control genome stability in breast cancer through the early onset gene *BRCA1*, one group at the National Cancer Institute has provided one of the first comprehensive genomic analyses of the interaction of CtBP with the human genome [185]. Using a combination of chromatin immunoprecipitation and deep sequencing (ChIP-seq), this group identified more than 1800 gene promoters that were potential candidates for transcriptional regulation by CtBP [185]. Remarkably, many of the genes that were targets of CtBP repression fell into 3 major classes: those genes that influenced genome stability (e.g., *BRCA1, BRIP1, RAD51C, ERCC5, PALB2, FANCD2, XRCC5*, and *FANCM*); those genes that controlled epithelial differentiation and are therefore down regulated during EMT (e.g., *CDH1, CST6, CLDN3, CLDN7, CLDN9, GRHL2, KRT18*, and *PARD6B*); those genes that are routinely repressed to maintain stem-cell like self-renewal and pluripotency (e.g., *HES1, OVOL2, FOXA1, GATA3, DKK1, CEBPb, RARG,* and *OAZ3*) [185] (see Figure 1). This was an important finding since these pathways represent significant hallmarks of cancer that play major roles in more aggressive forms of cancer through the promotion of uncontrolled growth, resistance to chemotherapy, and invasion and metastasis [186, 187]. As mentioned earlier, breast cancer subtypes stratify by morphology, molecular attributes, and prognosis along a hierarchy that reflects normal mammary epithelial development [188–190]. Tumors with high levels of phenotypic plasticity characterized by primitive, embryonic, or dedifferentiated mesenchymal features are usually of the basal-like and claudin-low subtypes. These tumors are typically estrogen receptor negative and the majority are also negative for the progesterone receptor and the human EGF receptor 2 (HER2) [189–191]. Such receptor negative tumors are often referred to as triple negative breast cancer (TNBC). These tumors also have the worst clinical outcomes with high mortality within the first five years after diagnosis [189–192]. Key molecular attributes of these tumors are increased genome instability, early invasion and metastasis, resistance to chemotherapy, and high expression of stem cell-like self-renewal pathways [189–194]. Strikingly, the CtBP target genes identified in this study could be readily used as a "signature" to predict poor clinical outcome based on metastasis free interval [185]. Moreover, using human breast cancer cell lines, the authors showed that disruption of CtBP, by either gene depletion or calorie restriction to lower endogenous NADH levels, increased DNA repair and diminished both the stem cell-like and invasive attributes of the cancer cells thus establishing a clear mechanistic link between CtBP, cellular metabolic status, and aggressive features of breast cancer. Finally, a screen of clinical samples from breast cancer patients revealed that those patients that had the highest expression of CtBP in their tumors had substantially shortened median breast cancer survival [185].

14. Conclusion

In this short review, we have described how, NADH, a central product of carbohydrate metabolism can act as a secondary messenger to control the activity of multiple different epigenetic regulatory complexes in human cells and how these modes of regulation, when disrupted by metabolic imbalance, can increase the risk of cancer of the breast and other types. The linkage in metabolism and epigenetic modification provides a novel window through which one could assemble newer strategies for therapy with particular focus on the nexus between metabolism and epigenetic modifiers. In this regard, it is reasonable to conjecture that certain metabolic therapies could have the potential to show efficacy in combination with epigenetic modalities in the treatment of breast and other cancers. The heightened excitement raised about the reported efficacy of the antidiabetic drug, metformin, as both a chemopreventive and treatment strategy in breast cancer is just one example [195, 196]. Other examples include the use of metabolic transducers like CtBP as a biomarker for efficacy in epigenetic therapy [179]. Since a direct effect of metformin is to increase AMPK activity, the finding that AMPK inhibits CtBP activity [93] certainly lends credence to such notion. Thus, new strategies to search for potential small molecules that may disrupt CtBP function could represent

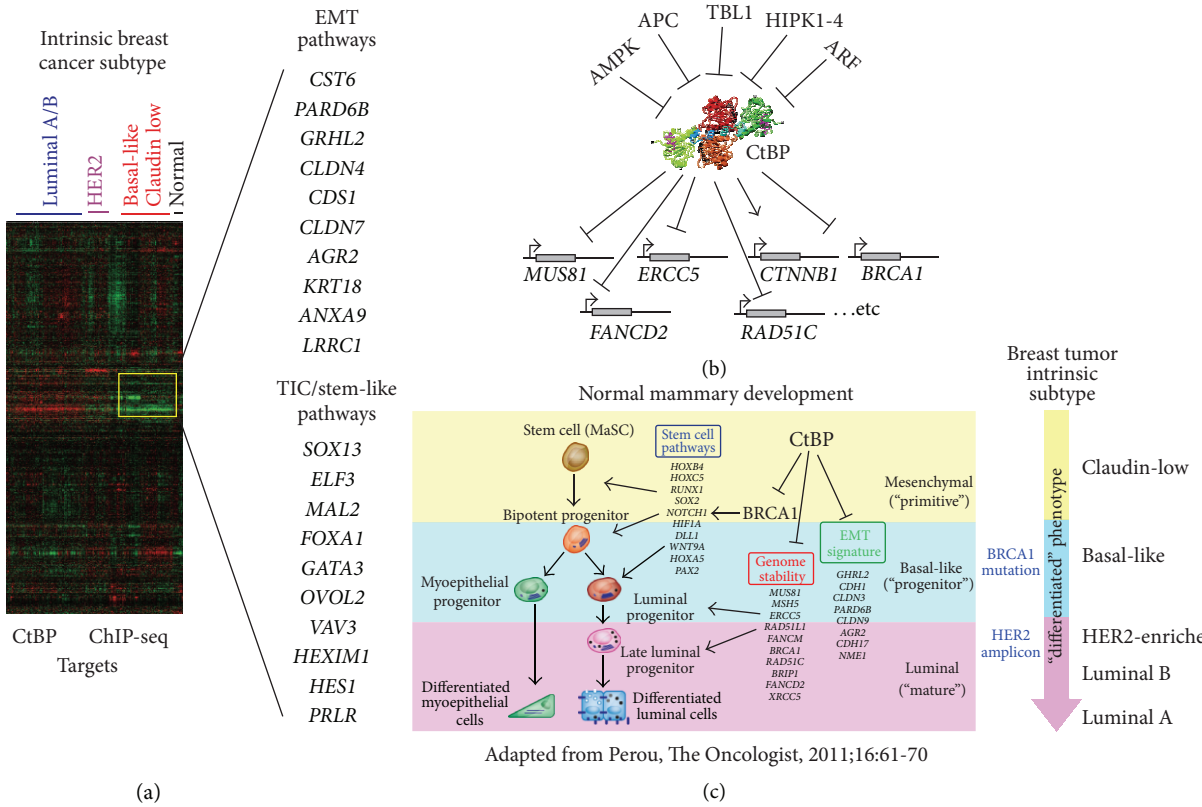

Figure 1: CtBP targets a network of interactions that control cellular reprogramming. (a) ChIP-Seq signature identifies multiple genes that are downregulated in breast cancer subtypes with primitive and mesenchymal features including the basal-like and claudin-low subtypes. (b) The CtBP targeted genes represent a network that exerts transcriptional control at the level of gene promoters and posttranslational stability of CtBP. (c) Representative genes targeted by CtBP influence cellular programming that correlate with primitive and more clinically aggressive intrinsic subtypes of breast cancer.

a novel form of epigenetic therapy where the target is gene-specific recruitment of chromatin modifying enzymes rather than the wholesale nonspecific repression of a whole enzyme class.

While we specifically focus on CtBP, a pleiotropic regulatory complex controlling numerous epigenetic modifications, whose activities are "metabolically transduced" by NADH, there are clearly many other metabolic intermediates that influence epigenetic modifications and therefore provide a means through which aspects of metabolic status can be transduced to affect changes in gene expression through epigenetic regulation. Among them are the wide array of histone acetyl-transferases that utilize acetyl-CoA for producing epigenetic marks, the PARP family of proteins that modify chromatin by ADP-ribosylation, the Sirtuin family that consumes NAD+ during histone deacetylation, the Jumonji C family of histone methyl-transferases that utilize alpha-ketoglutarate, and the TET1/2 family of 5 mC oxidases that also consume alpha-ketoglutarate to influence DNA methylation. The manner in which the activities of these epigenetic regulators are coordinated with each other to sculpt and shape the epigenome in response to cellular metabolism represents a new area of investigation that will have broad implication not only in cancer but in a wide variety of human diseases.

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Review Article

Natural Compounds as Regulators of the Cancer Cell Metabolism

Claudia Cerella,[1] Flavia Radogna,[1] Mario Dicato,[1] and Marc Diederich[2]

[1] *Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg 9, Rue Edward Steichen, 2540 Luxembourg, Luxembourg*
[2] *Department of Pharmacy, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea*

Correspondence should be addressed to Marc Diederich; marcdiederich@snu.ac.kr

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Even though altered metabolism is an "old" physiological mechanism, only recently its targeting became a therapeutically interesting strategy and by now it is considered an emerging hallmark of cancer. Nevertheless, a very poor number of compounds are under investigation as potential modulators of cell metabolism. Candidate agents should display selectivity of action towards cancer cells without side effects. This ideal favorable profile would perfectly overlap the requisites of new anticancer therapies and chemopreventive strategies as well. Nature represents a still largely unexplored source of bioactive molecules with a therapeutic potential. Many of these compounds have already been characterized for their multiple anticancer activities. Many of them are absorbed with the diet and therefore possess a known profile in terms of tolerability and bioavailability compared to newly synthetized chemical compounds. The discovery of important cross-talks between mediators of the most therapeutically targeted aberrancies in cancer (i.e., cell proliferation, survival, and migration) and the metabolic machinery allows to predict the possibility that many anticancer activities ascribed to a number of natural compounds may be due, in part, to their ability of modulating metabolic pathways. In this review, we attempt an overview of what is currently known about the potential of natural compounds as modulators of cancer cell metabolism.

1. (Re-)Evaluating the Targeting of Metabolic Alterations in Cancer

Deregulated metabolism is one of the oldest mechanisms associated with cancer physiology. The actual meaning and the selective advantages induced by this deregulation remain nowadays still a matter of debate despite the pioneering work of Warburg about the impact of the alteration of the energetic metabolism in cancer cells. Certainly, several reasons have significantly contributed to delay the advancement in this area of investigation. For many years, the search for new anticancer therapeutic agents has been extremely focused on fighting the two most intuitive altered features of cancer cells, namely, their sustained and uncontrolled proliferation and their ability of evading death. Accordingly, we have assisted over the years in the development of different classes of therapeutic agents reducing cancer cell proliferation or inducing cancer cell death. The main target of these studies was the differential susceptibility of cancer versus normal cells to these treatments. Over the time, however, we have also learned about the limits of this approach considering the high incidence of therapeutic failure and the frequent development of systemic toxicity.

Recently, the high level of complexity and heterogeneity of cancer allowed considering this disease as a dynamic multicellular system with complex forms of interactions and cellular communications with the own environment. It has become evident that consolidated cancer hallmarks including sustained and uncontrolled cell proliferation and resistance to cell death need to be reconsidered in a much more complex modulatory context if we want to therapeutically succeed with cancer.

At the light of this new vision, the ability of cancer cells to reprogram their cellular energetic metabolism is passing through a renaissance of interest in cancer biology for these chapters of fundamental biochemistry. The discovery

of unexpected cross-talks between well-known metabolic factors and mediators of unrelated processes is fuelling this renewed interest. On one side, noncanonical regulatory functions of specific metabolic enzymes or substrates are emerging; on the other side, oncogenes, tumour suppressors, as well as modulators controlling events typically altered at the very early stages of cancer progression including immune response, cell proliferation, or cell death appear in the dual role of controlled/controllers of metabolic processes. Decoding the roles of metabolic changes occurring during carcinogenesis and identifying the key nodes that differentiate pathological and healthy behavior have two important implications: novel predictive biomarkers and new drug discovery strategies. Consequently, additional knowledge may offer new tools to troubleshoot frequent chemotherapeutic failures; additionally, compounds targeting metabolic processes may also be potentially used for chemopreventive purposes. This research is only emerging, transforming the identification of metabolically active agents into an opportune challenge.

Nature provides a considerable source of biologically active compounds with a diversified pharmacological potential. Remarkably, almost 80% of all anticancer compounds are isolated from plants, fungi, and microorganisms. Both natural and chemically modified molecules (in order to improve stability, specificity, and/or activity) are able to counteract each of the cancer hallmarks [1, 2] recently reclassified by Hanahan and Weinberg [3]. Accumulating evidence also concerns cancer metabolism [2]. Remarkably, many of these compounds are food constituents or have been used since a long time in traditional medicine. Thus, they show a favorable profile in terms of their absorption/metabolism in the body with low toxicity.

2. Advantages of Altered Metabolism in Cancer versus Normal Cells

2.1. Metabolic Switch from Mitochondrial Respiration to Glycolysis. The preferential switch from oxidative phosphorylation to aerobic glycolysis represents the most discussed and investigated altered metabolic feature of cancer cells and was first described by Otto Warburg in the 1920s. He already hypothesized mitochondrial dysfunctions as the causative event. Defects in the enzymatic respiratory chain exist in cancer cells [4]; however, there is no clear correlation between the incidence of mitochondrial dysfunctions and the metabolic switch to glycolysis, the latter being commonly reported in cancer cells. In a number of instances, instead, cancer tissues/cells even consistently rely on mitochondrial respiration to produce ATP [5]. Furthermore, under specific circumstances, cancer cells may also be forced to reactivate mitochondrial energy production [6]. These observations clearly show that mitochondria are generally functional in cancer cells and support the hypothesis that the propensity of cancer cells to exacerbate the glycolytic pathway, while decreasing oxidative phosphorylation, must be an active option conferring important advantages despite the evident energetic inefficiency of glycolysis. Nevertheless, identification of these selective advantages is not an obvious task, being indeed matter of debate.

Theoretically, metabolic alterations during carcinogenesis could provide multiple benefits as cancer cells need to satisfy a continuous demand in macromolecule precursors to maintain their high proliferation rate. As a matter of fact, the reduction of mitochondrial respiration prevents a complete degradation of glucose to carbon dioxide (CO_2) and water and leads to accumulation of precursors used by the major cellular synthesis pathways leading to amino acids, nucleotides, and lipids. Consequently, this metabolic alteration inevitably fuels these anabolic pathways. Second, cancer cells experience moderate to severely reduced oxygen tension, and the fact to preferentially exploit glycolysis to produce energy in this situation represents an interesting adaptation. Accordingly, overexpression or stabilization of the hypoxia-inducible factor (HIF) in response to low-oxygen conditions promotes the glycolytic metabolism, by inducing transcription of glucose transporters and numerous key glycolytic enzymes [7].

An increased glycolytic flux means also very frequently overexpression and/or increased activity of specific isoforms of several glycolysis-related enzymes. Glucose transporters, or key enzymes as hexokinase II (HKII), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH) and the isoform M2 of pyruvate kinase (PKM2) are upregulated in cancer cells and accordingly suggested as potential therapeutic targets [8, 9]. Interestingly, nonglycolytic functions are also emerging for several of these enzymes, and the novel activities ascribed do further promote cancer aggressiveness. For example, GAPDH, LDH, or PKM2 may additionally activate gene expression by working as direct transcriptional factors or by interacting with and, thereby, modulating the activity of other nuclear proteins [10–12] (including HIF-1 and the Signal Transducer and Activator of Transcription 3 (STAT3) [13, 14]) required in the transcription of genes especially implicated in cell proliferation (e.g., histones H2A and H2B, MEK5, c-Myc, cyclin D1, and androgen receptor [10, 13–15]).

The hyperproduction of lactate plays a dual role. On one side, it activates the glycolytic pathway, ensuring the regeneration of nicotinamide adenosine diphosphate (NAD^+), as part of a feedback regulatory mechanism; on the other side, it is secreted outside the cells where it promotes angiogenesis and spreading of cancer cells from their primary site. A mutual control exists between events controlling lactate production and synthesis of proangiogenic factors. For example, the extracellular acidification due to the transport of lactate coupled to H^+ extrusion promotes upregulation of HIF-1 [16, 17]. HIF-1, in turn, transactivates the LDH-A promoter [16]. Besides, acidic conditions destabilizes the behavior of the immune system, which further contributes to cancer invasion. Lactate secretion, indeed, impairs the function of specific immune cells (including cytotoxic T lymphocytes) and cytokine production [18]. Furthermore, it promotes cell motility by controlling the expression level of constituents of the matrix [19, 20].

Identifying further advantages of the Warburg effect, other intriguing explanations involve mitochondria. Reducing the mitochondrial metabolism may inevitably decrease accumulation of reactive oxygen species (ROS). Suppression of ROS formation has been suggested as an important advantage for rapidly proliferating cell systems; these cells may be better protected against the risk of DNA damage during DNA synthesis [21]. This model seems to be encouraged by the observation that healthy highly proliferating systems temporarily switch to glycolysis before entering in S-phase [22]. Moreover, c-Myc, which activates transcription of the glycolytic enzymes HKII, enolase-1 (ENO-1), and LDH (subunit A), further promotes this switch in concomitance with the entry in S-phase [22–24].

More recently, unconventional roles were ascribed to pyruvate, the end product of glycolysis, which is massively converted into lactate in cancer cells instead of being transported into mitochondria to initiate mitochondrial metabolism. The plasma membrane transporter SLC5A8 was reported to be downregulated in different human cancer cells [25, 26]. Its silencing occurs at very early stages of carcinogenesis; moreover, the restoration of its expression triggers cell death [27]. Accordingly, it has been hypothesized that SLC5A8 may act as a tumour suppressor. This transporter couples Na^+ extrusion to the intake of extracellular monocarboxylates, including pyruvate, into the cell. The group of Ganapathy has proposed an interesting model to explain the tumour suppressor activity of SLC5A8 specifically centered on the role of pyruvate [28]. According to their findings, pyruvate acts as a specific inhibitor of histone deacetylase-(HDAC-)1 and -3 isoforms, an event that in turn promotes cell death [28]. Therefore, keeping low levels of pyruvate may stabilize specific epigenetic aberrations established in cancer cells and promote cancer cell survival. Remarkably, pyruvate is maintained at very low levels in cancer cells [27]. Accordingly several mechanisms may participate in buffering the intracellular pyruvate levels together with upregulated LDH-A in cancer cells. They include also transporters as SLC5A8 and, conceivably, other monocarboxylate-specific transporters whose expression is modulated in cancer cells [29]. This model has fascinating implications. It assigns to pyruvate itself the role of a tumour suppressor [27]. Therefore, the control of intracellular pyruvate levels could play an active and central role in the altered metabolic profile of cancer cells. Moreover, it prompts us to consider additional roles for typical altered metabolic conditions in cancer cells that deal directly or indirectly with pyruvate accumulation. The preferential expression of the less efficient dimeric form of PKM2 (slowly accumulating pyruvate) or the relevance of the exacerbated conversion of pyruvate into lactate would be two interesting conditions to further investigate. In addition, these considerations remind us how much each metabolic alteration in cancer may play multiple functions, well exploited by cancer cells to succeed and ultimately survive and proliferate.

2.2. Relevance of Other Altered Metabolic Pathways in Cancer. Preferential exploitation of aerobic glycolysis by cancer cells is a key issue of reprogrammed metabolism. It is becoming clear that other metabolic pathways or mediators may play a fundamental role in cancer. The availability of recent sophisticated experimental approaches to study the metabolic profile of cancer cells has allowed identification of an impressive number of alterations. They essentially concern levels of expression/accumulation or status of enzymes or intermediate substrates involved in several anabolic pathways. Despite the evident advantage of these modifications within the anabolic process in which they are mainly involved, additional noncanonical functions have emerged, including control of redox homeostasis or specific signalling events enabling the high cellular proliferation rate. In this section, we will briefly discuss two key pathways suitable for therapeutic targeting.

2.2.1. Glutamine Metabolism. Beside glucose, cancer cells frequently rely on glutamine metabolism. This amino acid is uptaken through specific transporters and directed to the mitochondria where it is converted first in glutamate (by a mitochondrial glutaminase). Glutamate then fuels the tricarboxylic acid cycle (TCA), upon further conversion to α-ketoglutarate in a reaction catalyzed by glutamate dehydrogenase (GDH). Exceeding substrates from the TCA cycle can be again available in the cytosol where they become the precursors of several anabolic pathways leading to biosynthesis of lipids, other aminoacids, and nucleotides. Beside its relevant role in anabolic pathways, glutamine metabolism may also promote further accumulation of lactate (via malate formation) and therefore exacerbate glycolysis and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) generation (glutaminolysis), the latter further buffering potential oxidative stress into the cells. Studies highlight that specific forms of cancer including glioblastoma develop an impressively high rate of glutamine metabolism, which goes beyond the real nitrogen demand, thus suggesting that glutamine consumption in cancer cells may represent a fast and preferential carbon source to replenish several biosynthetic pathways [30]. This preferential use of glutamine may be further promoted by other factors, whose expression level is altered in cancer cells, for example, the NFE2-related factor (NRF2) [31]. Altogether these observations imply that cancer cells may become addicted to glutamine metabolism to maintain their high rate of proliferation. Therefore, targeting their ability to degrade glutamine may be of therapeutic relevance especially in glutamine-dependent types of cancer.

2.2.2. Lipid Metabolism. A growing body of evidence depicts a determinant role of altered lipid homeostasis in enabling the cancer cell phenotype. The pattern of alterations described suggests that lipid metabolism plays a multitasking role in cancer. Beyond the relevance of metabolic modifications that promote lipogenesis and therefore specific anabolic activities, lipid-related factors appear essential in controlling redox homeostasis and accumulation of specific lipid messengers, including lysophosphatidic acid and prostaglandins. Accordingly, several enzymes and transcription factors controlling

lipogenesis and lipid homeostasis are overexpressed in cancer, as we will detail later. These alterations were initially identified in hormone-dependent malignancies such as those affecting breast [32] and prostate [33], thus confirming the relevance of steroid hormone-dependent pathways in the observed altered lipid metabolism. More recently, comparable patterns of alterations were identified in other cancer cell lines derived from melanoma [34], osteosarcoma [35], colorectal [36, 37], and lung cancer [38], as well as in hematopoietic cancer cells [39, 40]. These cellular environments allowed to identify additional modulatory upstream pathways including mitogen-activated protein kinase-(MAPK-) dependent [41], phosphatidylinositol-3-kinase (PI3K)/Akt pathway [41, 42], H-ras [41] and AMP-activated protein kinase, AMPK [43]. In addition, a lipid-related transcription factor, the sterol regulatory element-binding protein (SREBP), whose target genes promote cancer aggressiveness [44], is upregulated in cancer.

It is well-known that fatty acid neosynthesis is triggered by excess glucose leading to increased mitochondrial citrate concentrations. Citrate is then converted in the cytoplasm into palmitoyl-CoA, the precursor of triglycerides, and phospholipids synthesis. Accumulation of triglycerides may be reverted after starvation when a decrease of the lipogenic intermediate malonyl-CoA reactivates carnitine palmitoyltransferase-1 (CPT-1), thus leading to mitochondrial fatty acid oxidation [45].

In cancer cells, *de novo* fatty acid synthesis is sustained and several lipogenic enzymes are typically upregulated. The consequent burst in lipidogenesis confers the advantage to further exacerbate additional biosynthetic anabolic activities enabling cell growth. The enzymes ATP-citrate lyase (ACL), acetyl-CoA carboxylase (ACC), and the fatty acid synthase (FAS) are frequently overexpressed in cancer cells [46]. Especially FAS was described as a potential cancer biomarker [47, 48] for therapeutic purposes [49]. This dual clinical potential is supported by the observation that FAS inhibitors suppress carcinogenesis in *in vivo* procarcinogenic models of breast [50] and lung [38] tissues; moreover, they trigger cell death in a number of cancer cell lines [34, 47, 51–53], without affecting normal lipogenic tissues [54]. Additionally, FAS expression correlates with metastasis formation [35] and its targeting alleviates chemoresistance when combined with chemotherapeutic agents [55]. These multiple anticancer activities together with the observation that FAS are overexpressed in premalignant lesions [56, 57] strongly point at a very early role of FAS overexpression in carcinogenesis and led to the speculation that this enzyme may effectively be considered an oncogene [58–60].

Besides, cancer cells show a preferential synthesis of phospholipids (i.e., lysophosphatidic acid) instead of triglyceride [49]. This biosynthetic diversion of lipid precursors leads to the accumulation of lipid messengers regulating a number of signalling events promoting cancer cell growth, survival and migration to other tissues [61]. An accumulation of prostaglandins (i.e., prostaglandin E) strengthens the procarcinogenic roles played by proinflammatory signalling events during carcinogenesis [62]. Remarkably, a tight crosstalk exists between lipid metabolism and modulation of the expression of the main proinflammatory mediator cyclooxygenase 2 (COX-2), which is constitutively overexpressed in cancer [62, 63]. In line with these observations is also the fact that lipolytic enzymes like the monoacylglycerol lipase (MAGL) [64] are overexpressed in cancer and may directly control the prostaglandin levels [65].

Taking into account recent publications about the roles of lipid metabolism in cancer, we are convinced that further discoveries will further strengthen the importance of these pathways in cancer treatment and prevention.

2.3. Role of Altered Metabolism in Promoting Specific Cancer Hallmarks. Cell death resistance and angiogenesis are two important pathways involved in tumour progression and survival [66–68]. These independent processes are closely linked to cancer cell metabolism [67, 69]. Recent publications highlight mitochondria as modulators of these two critical pathways and promoters of metabolic homeostasis in cancer cells [70]. The mitochondrion is the most important coordinator of both energy production and accumulation of biosynthetic precursors for cellular maintenance and survival.

Altered mitochondrial bioenergetics and functions play an important role in tumorigenesis by affecting cancer cell metabolism, decreasing mitochondria-dependent apoptosis, and contributing to angiogenesis [66, 70–72]. Cancer cells present frequently a mitochondrial metabolic shift from glucose oxidation (GO) to glycolysis, thus assimilating a larger amount of glucose compared to normal cells [73]. By this way, cancer cells refuel themselves with phosphorylated intermediates required for growth and proliferation, through regulation of the metabolic key enzymes that govern the balance between GO to glycolysis and by reducing the entry of pyruvate into mitochondria thus reducing the rate of TCA cycle [17, 73, 74]. The accumulated pyruvate is in part converted to lactate during aerobic glycolysis and secreted to keep glycolysis active. The extracellular secreted lactate influences the extracellular matrix lowering the pH of the tumour environment, allowing a remodelling of the matrix and inducing blood vessel invasion in response to tumour-induced angiogenic factors [17]. Therefore, the reduced mitochondrial efficiency may induce the activation of HIF-1α resulting in angiogenesis activation, cell migration, increased cell survival, and energy metabolism [75, 76]. Conversely, restoration of the mitochondrial activity inhibits HIF-1α [77–79]. It has been demonstrated that dichloroacetate (DCA), which inhibits pyruvate dehydrogenase kinase (PDK), activates GO in mitochondria thus leading to decreased tumour growth in many cancer cell lines; this event is accompanied by the inhibition of HIF-1α [69].

Alterations in mitochondrial function not only influence the cellular metabolic status but also contribute to the control of the redox status of cancer cells. The large amounts of glucose available in the cells are metabolized through the pentose phosphate pathway (PPP) producing nucleosides and generating NADPH [70, 73].

NADPH is essentially involved in redox control protecting cells against ROS. High levels of ROS, as generated in cancer cells, can promote oxidative damage-induced cell death.

Therefore, cancer cells maximize their ability to produce NADPH to reduce ROS activity [73]. The difference in the redox status between normal and cancer cells may be a target to selectively kill cancer cells by ROS-generating drugs. Thus, the elicitation of ROS can be exploited to induce cancer cells to undergo oxidative damage-induced cell death.

Another important modulator of the redox status in cancer cells is B-cell lymphoma-2 (Bcl-2) protein that is, overexpressed in a variety of cancer cells [80]. The potential tumorigenic activity of Bcl-2 is due to its antiapoptotic properties maintaining the integrity of the outer mitochondrial membrane and preventing its permeabilisation through sequestration of the proapoptotic protein B-Cell lymphoma-associated X (BAX) and Bcl-2 homologous antagonist killer (BAK). However, regulation of ROS levels by Bc-2 was also demonstrated [81, 82] as Bcl-2 may affect the intracellular redox status in order to maintain the ROS potential at the most favorable level for cancer cell survival.

Autophagy is another alternative pathway that sustains tumour cell survival. Moreover, autophagy is a major process fueling cell metabolism [67]. It supplies intracellular nutrients when the external ones are not available. Unlike normal cells, cancer cells are placed in an environment deprived of nutrients and oxygen due to an insufficient vascularization. Autophagy may support tumour growth ensuring the availability of endogenous metabolic substrates necessary to feed glycolysis, ATP production, and pyruvate for the mitochondrial metabolism [67]. Autophagy recycles intracellular organelles and the resulting breakdown products contribute to produce energy and build up new proteins and membranes. Indeed, autophagy provides an internal source of sugar, nucleosides, amino acids, and fatty acids by the degradation of protein, lipids, carbohydrate, and nucleic acids [83]. Thus, autophagy sustains cell metabolism and subsequently favors cancer cell survival in nutrient lacking tumours, besides preventing that cancer cells may accumulate dysfunctions in their mitochondria [84].

Impaired mitochondrial functions, oxidative stress, and autophagy are tightly correlated. Emerging evidence underlines how much autophagy may affect mitochondrial functions and accumulation of ROS [85]. Number and the health status of mitochondria are controlled by an autophagic process called mitophagy. Mitophagy is a mitochondrial quality control by means of which excessively damaged mitochondria become a substrate for autophagic degradation. Hypoxia and hypoxia-inducible factors (HIFs) can induce mitophagy [86]. Dysfunctional mitochondria are linked to ROS generation, induction of DNA damage, and cell death [87]. Thus, degradation of these defective organelles by mitophagy may protect cells from carcinogenesis. However, both activation and inhibition of the autophagic pathways may play a role in cancer therapy. It has been demonstrated that inhibitors of autophagy may target autophagy-dependent cancer cells because this modulation inevitably impairs cancer cell survival [88]. On the other side, an excessive autophagic flux can induce cell death. Therefore, cytotoxic cancer therapies exacerbating autophagy may provoke increased oxidative stress or severe cell damage, thus sensitizing cancer cells to cell death (i.e., apoptosis) [89]. Interestingly, autophagy and apoptosis are both regulated by Bcl-2. Bcl-2 regulates autophagy by binding to the proautophagy protein Beclin-1 and the proapoptotic protein Bax [72]. Therefore, the crosstalk between autophagy and the mitochondrial metabolism is an important issue to be considered for cancer therapy. Moreover, redox alterations associated with mitochondrial dysfunctions may be pivotal in preventing cancer formation, growth, and establishment at very early steps of carcinogenesis.

3. Potentially Targetable Metabolic Actors by Natural Compounds

The logical consequence of the elucidation of the multiple roles played by altered metabolism in cancer is the exploitation of this knowledge for preventive and therapeutic purposes. The existence of specific patterns of modulations identifies also potential molecular targets for future novel classes of anticancer compounds. In this section, we suggest an overview of natural compounds regulating the most interesting metabolic pathway intermediates.

3.1. Glycolysis-Related Factors

3.1.1. Glucose Transporters. It is essential for a cancer cell to activate the glycolytic pathway to satisfy the anabolic demand in consistent amounts of intracellular glucose. Glucose is carried into cells via specific plasma membrane transporters that lead to glucose internalization by facilitation or active coupling to ion fluxes like the extrusion of Na^+ [90].

Frequently, specific isoforms of glucose transporters are overexpressed in cancer cells. The facilitative glucose transporters (GLUTs) belonging to the solute carrier (SLC2) gene family are frequently overexpressed. Consistent data was published about isoforms 1, 3, 4, and 12. Therefore, targeting abnormal expression or activity of those carriers represents one promising strategy. Several natural compounds have been described as potential modulators of glucose transporters (Figure 1). A critical reading of the literature indicates that these compounds most likely affect expression of glucose transporters indirectly, rather controlling upstream modulatory mechanisms. This is also true for natural compounds. Annonaceous acetogenins are long chained fatty acid derivatives extracted from different tropical plants such as the tree *Annona muricata*, also known as Graviola. It has been recently shown that Graviola extracts exert multiple anticancer activities on pancreatic cancer cell models [91]. The extract reduces cell proliferation and viability by inducing necrosis; besides, it counteracts cell motility. The potential anticancer properties have been confirmed with mouse xenograft models, where Graviola extract reduces both tumour growth and formation of metastasis. An analysis centered on metabolic parameters underlines the ability of this compound to inhibit glucose uptake; besides, it strongly reduces the expression levels of several metabolic actors, including GLUT1 and GLUT4, HKII, and LDH-A. This pattern of modulation is the consequence of the modulation of multiple factors and pathways including the reduction of

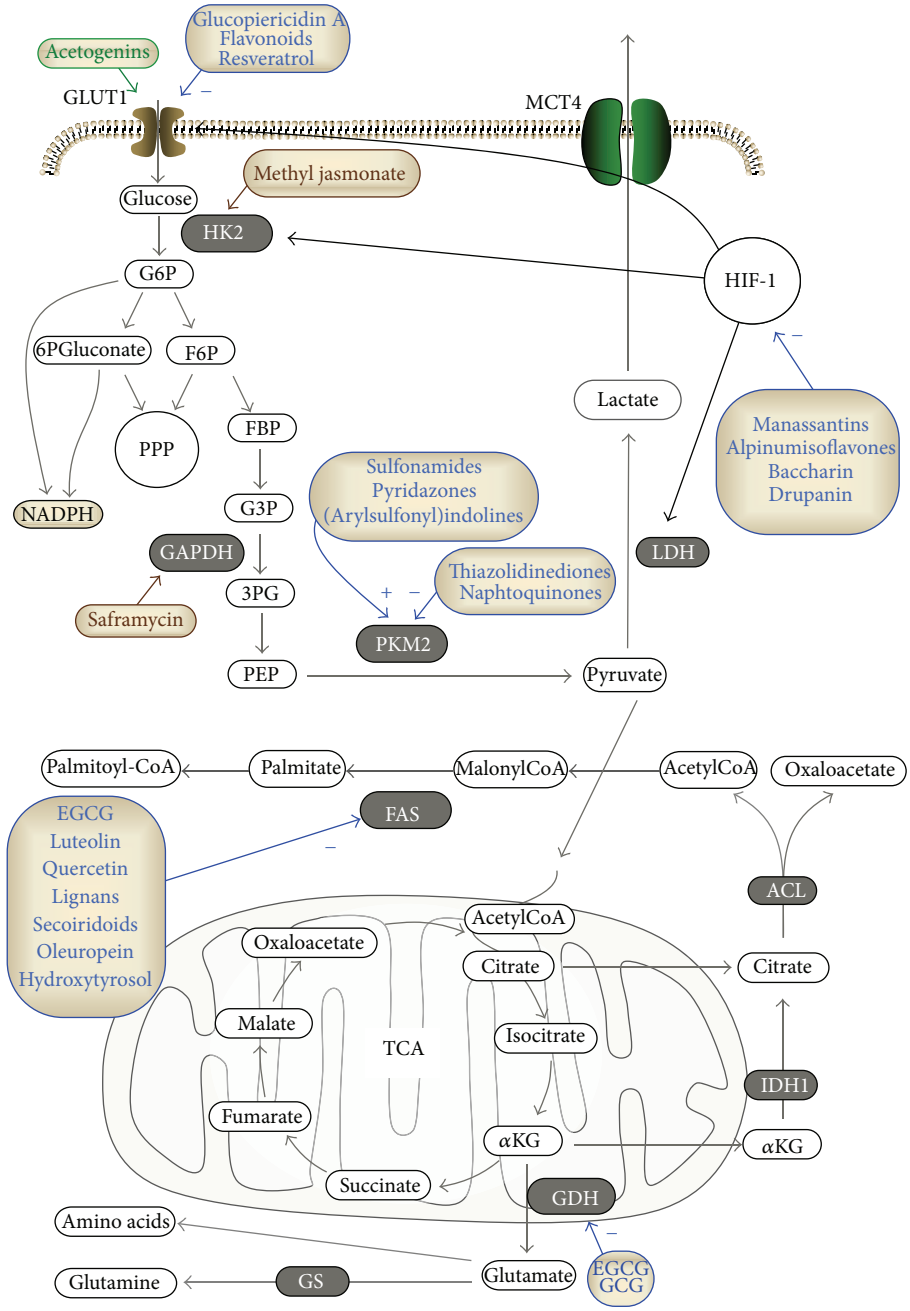

Figure 1: Targetable metabolic actors by natural compounds. A summary of the most relevant compounds affecting metabolic pathways of cancer cells. Many of these molecules correspond to natural compounds; alternatively, they are chemical structures found as active that may act as a template for the identification of promising natural compounds with similar activity. Molecules indicated in blue affect enzymatic activity (+ or − stands for activators or inhibitors, resp.); the ones in green affect the expression level of the targeted enzyme; the ones in brown affect nonmetabolic activities. Abbreviations: ATP citrate lyase, ACL; gallocatechin gallate, GCG; epigallocatechin gallate (EGCG); fatty acid synthase, FAS; fructose-6-phosphate, F6P; fructose-1,6-biphosphate, FBP; hypoxia-inducible factor 1, HIF-1; glucose-6-phosphate, G6P; glutamine synthetase, GS; hexokinase II, HK2; glyceraldehyde-3-phosphate, G3P; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; glucose transporter, GLUT; glutamate dehydrogenase, GDH; α-ketoglutarate (αKG); isocitrate dehydrogenase 1, IDH1; lactate dehydrogenase, LDH; nicotinamide adenine dinucleotide phosphate-oxidase, NADPH; pentose phosphate pathway; 3-phosphoglycerate, 3PG; phosphoenolpyruvate, PEP; pyruvate kinase isoform M2, PKM2.

HIF-1 and nuclear factor κB (NF-κB) expression levels and the inhibition of ERK (extracellular-regulated kinase) and Akt activation.

Due to the difficulties of specifically targeting glucose transporter expression without affecting many other intracellular pathways, an interesting alternative is to identify molecules that modulate the activity of glucose transporters. In this context several natural compounds deserve attention.

Following a natural product screening assay based on crude extracts of microbial origin aimed at identifying new inhibitors of filopodia protrusion (special membrane structures involved in promoting metastasis), Kitagawa and colleagues have isolated and characterized in the broth of *Lechevalieria sp.* bacterial strain glucopiericidin A (GPA) as a novel inhibitor of glycolysis [92]. The authors showed that GPA specifically impairs glucose uptake into the cells. Accordingly, the compound impairs the accumulation of the nonmetabolizable tritiated glucose analog 2-deoxyglucose (DG) without affecting the key glycolytic enzyme HK. Their findings suggest that GPA may act by mimicking a GLUT1 substrate.

From plants, polyphenols are interesting bioactive anticancer molecules as several of them have been repeatedly reported to control glucose transporter activity in different cancer cell models; fisetin, myricetin, quercetin, apigenin, genistein, cyaniding, daidzein, hesperetin, naringenin, and catechin are well-known inhibitors of glucose uptake [93]. Investigations designated hexose and dehydroascorbic acid transporters including GLUT1 and GLUT4 [94, 95] as their targets. Comparative studies indicate that these compounds do not exhibit the same mode of action as they bind different domains of GLUT1. Genistein binds the transporter on the external face whereas quercetin interacts with the internal face [95]. The ability of these compounds to act as protein-tyrosine kinase inhibitors is currently considered as the main mechanism responsible for the modulation of the glucose uptake.

3.1.2. Glycolytic Enzymes. Hexokinase (HK) is the enzyme controlling the first enzymatic step of glycolysis, allowing intracellular transformation of glucose via phosphorylation (Figure 1). In cancer cells, HKII is the main isoform and is involved in the Warburg effect and in enhanced cell proliferation [96]. HK associates with the outer mitochondrial membrane in proximity of ATP molecules required for HK's enzymatic activity. The destabilization of this physical interaction negatively affects the overall cancer cell energetics; moreover, it dramatically perturbs mitochondria, triggering the release of cytochrome c and, subsequently, inducing apoptosis [97]. Some natural compounds have been described as promoting the detachment of HK from mitochondria. Methyl jasmonate is a plant stress hormone produced by many plants including rosemary (*Rosmarinus officinalis L.*), olive (*Olea europea L.*), or ginger (*Zingiber officinalis*); it binds to HK and perturbs its association with the voltage-dependent anion channel (VDAC) in cancer cells [98]. This event leads to overall energetic impairment; moreover, it promotes the release of cytochrome c from mitochondria, triggering apoptosis. Its use in combination with the antiglycolytic agent 2-deoxyglucose or chemotherapeutic agents is currently under investigation [98].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key glycolytic enzyme catalyzing the conversion of glyceraldehyde-3-phosphate to glycerate 1,3-biphosphate, accompanied by the generation of NADH. There is evidence that GAPDH may play multiple noncanonical functions implicated in cell growth and survival. The *bis*-quinone alkaloid saframycin, a bacterial product of fermentation, exhibits antiproliferative properties in both adherent and nonadherent cancer cell models. This compound possesses activities comparable to alkylating agents. The group of Myers has shown that saframycin may form a nuclear ternary complex with GAPDH and DNA [99] involved in the antiproliferative effect ascribed to this compound [99].

Recently, the embryonic isoform M2 (PKM2) is attracting interest for diagnostic and therapeutic purposes in cancer [5]. Enzymes of the pyruvate kinase family catalyze the final, rate-limiting, step of glycolysis, leading to the accumulation of pyruvate from phosphoenolpyruvate in an ATP-producing reaction. Cancer cells exclusively express the embryonic isoform M2 instead of adult M1. This switch is required for the maintenance of aerobic glycolysis [8]. Also rapidly proliferating cells selectively express PKM2. Importantly, PKM2 exists as a dimeric or a tetrameric form; the latter one efficiently catalyzes pyruvate formation whereas the dimeric form is nearly inactive. In cancer cells the dimeric form is the preponderant one. This paradoxical behavior is believed to further promote glycolysis and several anabolic activities.

Currently two main PKM2 targeting strategies are under evaluation. The first attempt consists in identifying compounds inhibiting PKM2. High-throughput screenings based on an enzymatic LDH assay to explore a compound library including molecules approved from the Food and Drug administration (FDA) and purified natural products have led to the identification of three potential chemical structures associated with a potential inhibitory activities on PKM2 [100]. Active compounds include thiazolidinediones and natural compounds belonging to the group of naphthoquinones: shikonin, alkannin, and their derivatives (extracted from different plants including *Arnebia sp*. and *Alkanna tinctoria*) have been recently shown as the most potent and specific inhibitors of PKM2 [101]. These compounds reducing lactate production and glucose consumption in cancer cells are also known to induce necroptosis [102]. However, the inhibitory effect on PKM2 is independent of their effect on cell viability, rather suggesting an impairment of the glycolytic metabolism. Even though PKM2 is crucial for cancer cell survival [101], there is a potential risk to affect also healthy PKM2-expressing cells. Subsequently, a second line of research currently aims at promoting the reactivation of PKM2 in cancer cells. The increase of tetrameric versus dimeric PKM2 isoform ratio abrogates the Warburg effect and may reactivate oxidative phosphorylation [103]. So far a few promising studies have been published identifying some chemical scaffolds as potential PKM2 activators. They include sulfonamides, thieno[3,2-b]pyrrole[3,2-d]pyridazinones, and 1-(sulphonyl)-5-(arylsulfonyl)indolines

that act as small-molecule allosteric modulators binding to a surface pocket of the enzyme, thus facilitating the association of different PKM2 subunits.

Although PKM2 targeting appears a promising area for drug discovery, research remains preliminary. Identification of first chemical scaffolds may be the basis for the discovery of structurally related natural compounds.

3.2. Hypoxia-Inducible Factor-1: The Hypoxic Rheostat. There is no doubt that HIF-1 is a central molecule in the control of the expression of glucose transporters and key glycolytic enzymes as well (Figure 1). Accordingly, an important strategy is the identification of small molecule inhibitors of HIF-1. Several attempts rely on cell-based assays with reporter gene constructs under the control of a HIF-1 response element. The group of Zhou has discovered and characterized novel HIF-1 inhibitors in (i) manassantins (manassantin B and 4-o-demethylmanassantin) extracted from the aquatic plant *Saururus cernuus* [104] and (ii) alpinumisoflavones (alpinumisoflavone and 4′-O-methyl alpinumisoflavone) isolated from the tropical legomaceous plant *Lonchocarpus glabrescens* [105]. These compounds inhibit hypoxia-induced HIF-1 activation; besides, they may affect the expression of HIF-1 and HIF-1 target genes including GLUT1 and/or VEGF. Similarly, the group of Nagasawa has identified the cinnamic acid derivatives baccharin and drupanin, extracted from the Brazilian green propolis as inhibitors of HIF-1-dependent luciferase activity [106]. They inhibit the expression of HIF-1 and its target genes (GLUT1, HKII, and VEGF); besides, they exhibit antiangiogenic effects.

3.3. Modulation of Mitochondrial Metabolism and Functions. Several natural compounds have been shown to be able to target mitochondrial metabolism and functions, besides affecting cell death and angiogenesis, both important pathways involved in cancer progression.

Curcumin is a natural compound extracted from *Curcuma longa*, widely used as a spice. Its anticarcinogenic and chemopreventive effects target mitochondrial metabolism and function inducing cell death and angiogenesis in a variety of cancer models [107]. In human colorectal carcinoma cells, curcumin induces mitochondrial membrane potential, induces procaspase-3 and -9 cleavage and apoptosis in a dose- and time-dependent manner accompanied by changes, and release of lactate dehydrogenase. It leads to cell cycle arrest in S phase, accompanied by the release of cytochrome c, a significant increase of Bax and p53 levels, and a marked reduction of Bcl-2 and survivin in LoVo cells [108].

Dimethoxycurcumin (Dimc), a synthetic analogue of curcumin, induces cell cycle arrest in S phase and apoptosis in human breast carcinoma MCF-7 cells by affecting mitochondrial dysfunction by oxidative stress. Accordingly, it was observed that DNA damage and apoptosis followed an induction of ROS generation and a reduction of glutathione levels [109]. Mitochondrial dysfunction was also witnessed by a reduction of the mitochondrial membrane potential and a decrease of the cellular energy status (ATP/ADP) by the inhibition of ATP synthase. Therefore, the mitochondrial dysfunctions correlated with changes in the expression of apoptotic markers like Bax and Bcl-2 [109]. Several studies indicated redox alterations as a causative mechanism implicated in mitochondrial dysfunction in cancer. Chen et al. published a novel pathway for curcumin regulation of the ROS-lysosomal-mitochondrial pathway (LMP) and identified cathepsin B (cath B) and cathepsin D (cath D) as key mediators of this pathway in apoptosis. In lung A549 cancer cells, curcumin induces apoptosis via lysosomal membrane permeabilisation depending on ROS increase, which precedes the occurrence of mitochondrial alterations [110]. Further studies demonstrated that curcumin-induced ROS generation decreases the mitochondrial membrane potential followed by downregulation of Bcl-2 expression, Bax activation, and release of cytochrome c into the cytosol, paralleled by the activation of caspase-9 and -3 in small cell lung cancer (SCLC) and NPC-TW 076 human nasopharyngeal carcinoma cells [111, 112].

Curcumin-induced apoptosis in the colon cancer cell line HCT116 is significantly enhanced by the suppression of mitochondrial NADP(+)-dependent isocitrate dehydrogenase activity which plays an essential role in the cell defense against oxidative stress by supplying NADPH for the antioxidant systems [113].

Amaryllidaceae alkaloid pancratistatin isolated from the bulb of *Hymenocallis littoralis* exhibits potent apoptotic activity against a broad panel of cancer cells lines with modest effects on noncancerous cell lines [114]. Pancratistatin led to ROS generation and mitochondrial depolarization, leading to caspase-independent cell death in breast carcinoma cells. In colorectal carcinoma cell lines, but not in noncancerous colon fibroblast cells, pancratistatin decreased mitochondrial membrane potential and induced apoptotic nuclear morphology independently on Bax and caspase activation [114]. In colon cancer cells, resveratrol, a natural stilbene from grapes, blueberries, or cranberries, induces apoptosis by nitric oxide production and caspase activation [115]. Conversely, in multiple myeloma cells resveratrol increased apoptosis, by blocking the activation of NF-κB and subsequently downregulation of target genes including interleukin-2 and Bcl-2, leading to cell cycle arrest [116].

The cross-talk between mitochondria and the autophagic machinery could be used as a therapeutic strategy. Resveratrol has several beneficial effects such as neuroprotection and cytotoxicity in glioblastoma cell lines. It has been demonstrated that resveratrol induced a crosstalk among autophagy and apoptosis to reduce glioma growth [117]. Indeed, resveratrol has an impact on the formation of autophagosomes in three human GBM cell lines, accompanied by an upregulation of autophagic proteins Atg5, beclin-1 and LC3-II [117]. However, the inhibition of resveratrol-induced autophagy triggered apoptosis with an increase in Bax expression and cleavage of caspase-3. Only the inhibition of both cell death pathways abrogated the toxicity of resveratrol. Thus, resveratrol activates autophagy by inflicting oxidative stress or cell damage, in order to sensitize glioblastoma cancer cells to apoptosis [117]. Also, curcumin treatment of human liver-derived HepG2 cells induces the reduction of mitochondrial membrane potential and the

activation of autophagy. Moreover, it has been demonstrated that curcumin activates mitophagy. This finding underlines the importance of mitophagy in the process of cell death of nasopharyngeal carcinoma cells [118].

As mentioned earlier, another important pathway in mitochondrial dysfunction involved in tumour progression is HIF-1α. It has been published that curcumin plays a pivotal role in tumour suppression via the inhibition of HIF-1α-mediated angiogenesis in MCF-7 breast cancer cells and in HepG2 hepatocellular carcinoma cells [119, 120]. Anticancer activity of curcumin is attributable to HIF-1 inactivation by Aryl hydrocarbon nuclear translocator (ARNT) degradation. Another natural compound with a potent antiangiogenic activity is the flavonoid bavachinin. Bavachinin inhibited increased HIF-1α activity in human KB carcinoma derived from HeLa cells [121]. In human HOS osteosarcoma cells under hypoxia, bavachinin decreased transcription of genes associated with angiogenesis and energy metabolism that are regulated by HIF-1, such as vascular endothelial growth factors (VEGFs), GLUT1, and HKII [121]. Bavachinin may be used as a therapeutic agent to inhibit tumour angiogenesis. Indeed, *in vivo* studies showed that injecting bavachinin significantly reduced tumour volume in nude mice with KB xenografts [121].

Figure 2 summarizes the major mechanisms of action described for natural compounds as mitochondrial modulators.

3.4. Targeting Other Altered Metabolic Pathways in Cancer Cells

3.4.1. Glutamine Metabolism. Glutamine and glucose are the main carbon sources used by cancer cells to satisfy their anabolic demand. Published data indicate a role for glutamine metabolism within the malignant cell phenotype. Accordingly, several cancer cell lines present a high rate of glutamine consumption and strategies are investigated to target enzymes implicated in this pathway. Inhibiting the activity of glutamate dehydrogenase (GDH) is an effective anticancer strategy as documented in glioblastoma cells with combinatorial treatments with agents depleting cells of glucose or inhibiting specific kinase-(i.e., AKT-) dependent pathways [122]. Polyphenols extracted from green tea including epigallocatechin gallate (EGCG) and catechin gallate (CG) inhibit GDH, by recognizing and binding to the site of the allosteric regulator ADP [123, 124]. These findings allow to speculate about the potential use of these polyphenols and of their derivatives with improved bioavailability in the treatment of glutamine-dependent forms of cancer.

3.4.2. Lipid Metabolism. FAS sustains the altered lipid metabolism in cancer cells. As discussed in Section 2.2.2, several reports support the relevance of this enzyme as a target in cancer cells. This enzyme is a complex system with seven different functional domains [125]. This property amplifies the possibility of impairing its enzymatic activity with different specific compounds.

Four major specific FAS inhibitors are known [126]. The antibiotic cerulenin (extracted from the fungus *Cephalosporium caerulens*) acts as noncompetitive inhibitor of the β-ketoacyl synthase domain [127]. Tetrahydrolipstatin, also known as Orlistat (a derivative of the natural compound lipstatin), targets the thioesterase domain of FAS [128]. Triclosan affects the enoyl-reductase activity of the enzyme [129]. Finally, the synthetic chemical derivative of cerulenin C75 is the most potent compound *in vitro* able to affect all the three domains mentioned earlier in a competitive irreversible way [129]. Orlistat was approved by the Food and Drug Administration (FDA) for its ability to reduce body weight. Besides, all these molecules display anticancer activities by blocking cancer cell proliferation and triggering cancer cell death [126]. Nevertheless, their actual application for cancer treatment is hindered by several side effects, which include their ability to modulate other enzymes (i.e., the increase of CPT-1 activity and fatty acid oxidation by cerulenin and C75 leading to weight loss [130, 131]), their reduced bioavailability (i.e., Orlistat [126]), or stability *in vivo* (i.e., C75 inactivation by intracellular glutathione and other small thiols [132]). Current research efforts focus on the design of new synthetic derivatives of this first group of molecules, on one side, and on the identification of new compounds of natural origin, on the other side, both potentially showing improved characteristics of specificity and bioavailability/stability *in vivo*.

In this context, the potential identification of new FAS inhibitor from natural compounds is a particularly interesting strategy, especially by investigating compounds of vegetal origin showing the double favorable profile of being regularly consumed in the diet and displaying at the same time hypolipidemic and anticancer activities. Several classes of polyphenols appear as very good candidates. Extracts from green and black tea have been repeatedly proved as lipidogenic inhibitors [133]. Further investigations have identified catechin gallate derivatives (including EGCG, epicatechin gallate (ECG), and catechin gallate (CG)) as specific FAS inhibitors as demonstrated by *in vitro* assays of FAS enzymatic activity [134, 135]. The galloyl moiety of the catechins is essential for the inhibitory activity of these molecules; it directly interacts and modulates the function of the β-ketoacyl reductase domain of FAS [134, 135]. The FAS inhibitory activity is common to other polyphenolic compounds. The group of Tian has first described several flavones including luteolin, quercetin, kaempferol, myricetin, fisetin, and baicalein as inhibitors of the β-ketoacyl reductase domain [136]. The flavone luteolin and the flavonols quercetin and kaempferol (and with a lower extent the flavone apigenin and the flavanone taxifolin) have been shown to act as potent inhibitors of lipogenesis in a comparative study with EGCG in prostate cancer [137]. An *in vitro* FAS enzymatic activity assay confirmed their ability to inhibit FAS, however, less potently compared to ECGC [137]. Tian and colleagues suggested that all polyphenolic FAS inhibitors share a biphenyl core potentially responsible for their described inhibitory activity [138]. Possible differences may account for a structure-dependent mechanism of action, where flavones as quercetin and kaempferol containing hydroxyl groups at specific positions [137] display a reversible fast binding inhibitory activity,

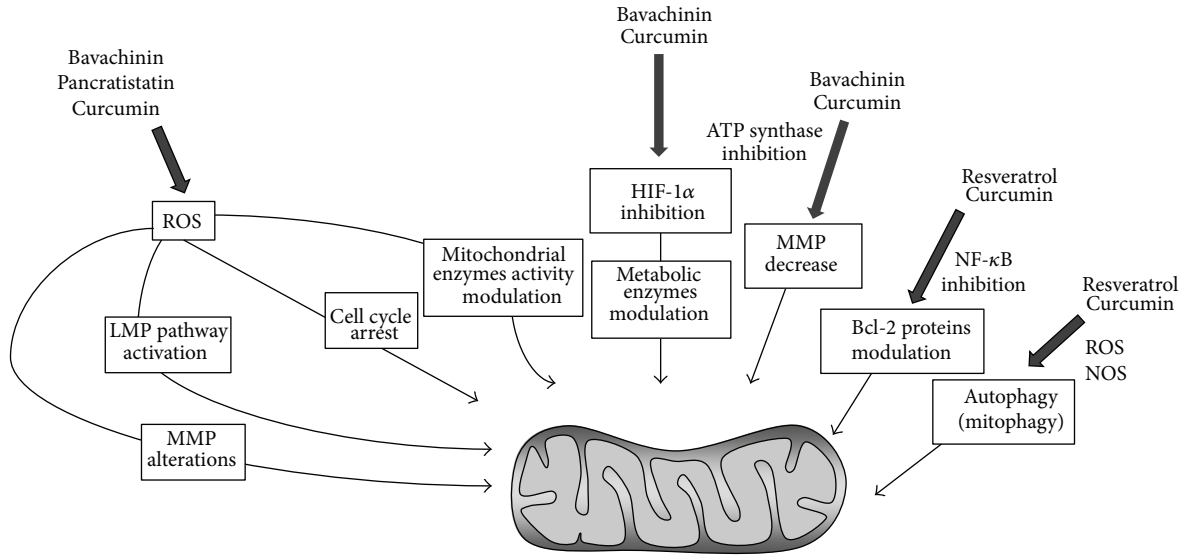

Figure 2: Mitochondrial dysfunctions as pharmacological targets. Examples of natural compounds with a potential efficacy in cancer treatment. The figure schematizes their mechanism of action linked to mitochondrial dysfunctions. The compounds discussed herein have different mitochondrial targets, such as mitochondrial membrane potential (MMP), Bcl-2 family proteins (Bcl-2), reactive oxygen species (ROS), HIF-1α, mitochondrial metabolism (MM), and autophagy.

whereas EGCG and ECG exhibit an irreversible slow binding activity [134]. It has been taken into account, however, that further variability may be associated with differential uptake, metabolization, and intrinsic stability of the compounds. Finally, the effects on lipid metabolism may be the result of multiple intracellular signalling events, modulated by polyphenolic compounds and eventually converging towards the control of the lipid metabolism. Curcumin has been shown to affect lipid accumulation and FAS activity [139]. This ability may be partially linked to the known antagonistic activity of this compound towards the NF-κB-mediated pathway [140]. Besides, curcumin and its derivatives have recently been shown to modulate the AMPK-SREBP pathway [141, 142]. Green tea extracts prevent EGF-induced upregulation of FAS in MCF-7 via modulation of a PI3 K/AKT-dependent pathway [143]. Other polyphenolic compounds have been identified as inhibitors of lipidogenesis by targeting FAS and/or the transcription factor SREBP expression through the modulation of specific pathways. These findings may therefore suggest further relevant pathways involved in the control of the lipid metabolism in cancer cells. For example, resveratrol, a stilbene contained in grapes, produces hypolipidemic effects by activating the NAD-dependent deacetylase sirtuin 1 (SIRT-1), which positively modulates AMPK [144]; AMPK activation, in turn, prevents lipid accumulation by controlling several events, including FAS downregulation [144]. The activation of AMPK by resveratrol has been also confirmed in other studies [145]. Moreover, it is a common property shared with compounds from other plants showing hypolipidemic properties, as observed with extracts from *Hibiscus sabdariffa* [146]. Promising interesting therapeutic implications may derive also from phenolic compounds contained in the extra-virgin olive oil, which was described as a very active inhibitor of FAS expression and controller of lipid biosynthesis in breast cancer cell models [147]. Compounds belonging to lignans (1-[+]-pinoresinol and 1-[+]-acetoxypinoresinol), flavonoids (apigenin and luteolin), and secoiridoids (deacetoxyoleuropein aglycone, ligstroside aglycone, oleuropein glycoside, and oleuropein aglycone) appear as the most active compounds, by activating AMPK and reducing SREBP-1 expression [147]. Similarly, polyphenols oleuropein and hydroxytyrosol from extra-virgin olive oil were able to inhibit FAS activity in colorectal cancer SW260 cells, and this effect correlated with their antiproliferative potential [148]. However, this effect could not be confirmed in another colon model (HT-29) suggesting cell-type specific effect and further unrelated mechanisms [148]. Targeting lipid metabolism and especially FAS activity remains a promising perspective to target cancer cell survival. Brusselmans and colleagues showed that palmitate added to the culture medium of prostate cancer cells allowed to bypass the downstream effects of FAS inhibition by luteolin on lipid metabolism and prevented the cytotoxic effect of this compound [137]; moreover, the silencing of FAS expression with FAS siRNA produced similar cellular alterations as luteolin [137]. These findings allow predicting a causative role of FAS inhibition in the antiproliferative and cytotoxic effect of polyphenols and prompt to explore the relevance of the control of the lipid metabolism by polyphenols in the anticancer activities ascribed to many of these compounds.

4. Concluding Remarks

The targeting of altered cell metabolism in cancer cell is a promising still unexplored area in anticancer strategies. In this review, we have highlighted that many of these modifications take place at very early steps of carcinogenesis, thus

at preneoplastic stages. Therefore, their targeting may be a powerful weapon for chemopreventive purposes. Besides, the literature clearly shows the crucial addiction of cancer cells to several metabolic aberrations to proliferate and survive, further underlining the importance of the targeting of some metabolic-related factors in future anticancer therapies.

Identification of specific aberrantly regulated metabolic keynodes, in terms of the expression and/or activity of these factors, delineates the nature of potential pursuable molecular targets. Despite all these considerations, the effective number of agents under investigations for antimetabolic purposes is still very poor and at a very preliminary stage. Good candidates should present a favorable profile ensuring excellent differential toxicity against cancer versus healthy cells, a reduced risk of systemic toxicity, combined to a favorable profile in specific pharmacological properties including bioavailability, half-life, and stability. Many natural compounds have so far been identified as anticancer agents by affecting almost each cancer hallmark [2]. Taking into account recently identified cross-talks between altered metabolic mediators and altered proliferation, survival, or migration properties, we may suspect that many of the anticancer properties so far ascribed to natural compounds are mainly due to a their potential in modulating cellular metabolism. Remarkably, we have reported here many examples of dietary polyphenolic compounds from fruits and vegetables, which display specific antimetabolic functions (Figure 1). Although there is consistent evidence of multiple beneficial biological properties on health, there are yet some obstacles which hinder promising natural compounds from being already used for chemopreventive and therapeutic purposes including bioavailability and adsorption. Moreover, information concerning the stability and the clearance of natural occurring compounds frequently remains to be yet determined; additional efforts will be required towards the elucidation of this important properties in the next future.

Nature represents an impressively huge "database" of different and diversified molecular scaffolds. Rapid advancement in new screening systems allowing the analysis of large libraries of isolated naturally occurring compounds offers new important and fast tools for the selection of promising antimetabolic compounds especially from dietary origins with reduced side effects. Relatively low costs for their extraction/production in large amounts make them interesting for commercial objectives and represent a good basis for chemical modifications that may further improve their anticancer activities and facilitate their pharmacological use and efficiency.

Abbreviations

AMPK: AMP-activated protein kinase
Atg5: Autophagy protein 5
ARNT: Aryl hydrocarbon nuclear translocator
Bcl-2: B Cell Lymphoma-2
BAX: B Cell Lymphoma-Associated X
BAK: Bcl-2 homologous antagonist killer
CO_2: Carbon dioxide
CG: Catechin gallate
COX-2: Cyclo-oxygenase-2
DCA: Dichloroacetate
ENO-1: Enolase-1
ERK: Extracellular-regulated kinase
FAS: Fatty acid synthase
HIF: Hypoxia-inducible factor
EGCG: Epigallocatechin gallate
GCG: Gallocatechin gallate
HKII: Hexokinase II
HDACs: Histone deacetylases
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GO: Glucose oxidation
GLUT: Glucose transporter
GDH: Glutamate dehydrogenase
LDH: Lactate dehydrogenase
LMP: Lysosomal-mitochondrial pathway
MAPK: Mitogen-activated protein kinase
MMP: Mitochondrial membrane potential
NAD: Nicotinamide adenosine diphosphate
NF-κB: Nuclear factor κB
NADPH: Nicotinamide adenine dinucleotide phosphate-oxidase
PPP: Pentose phosphate pathway
PI3K: Phosphatidylinositol-3-kinase
PDK: Pyruvate dehydrogenase kinase
PKM2: Pyruvate kinase isoform M2
ROS: Reactive oxygen species
STAT3: Signal Transducer and Activator of Transcription 3
SREBP: Sterol regulatory element-binding proteins
TCA: Tricarboxylic acid cycle
VEGF: Vascular endothelial growth factors.

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Research Article

Role of Insulin-Like Growth Factor Binding Protein-3 in 1, 25-Dihydroxyvitamin-D_3-Induced Breast Cancer Cell Apoptosis

C. Brosseau, G. Pirianov, and K. W. Colston

Division of Clinical Sciences, St George's University of London, London SW17 0RE, UK

Correspondence should be addressed to K. W. Colston; kcolston@sgul.ac.uk

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Insulin-like growth factor I (IGF-I) is implicated in breast cancer development and 1, 25-dihydroxyvitamin D_3 (1, 25-D3) has been shown to attenuate prosurvival effects of IGF-I on breast cancer cells. In this study the role of IGF binding protein-3 (IGFBP-3) in 1, 25-D_3-induced apoptosis was investigated using parental MCF-7 breast cancer cells and MCF-7/VD^R cells, which are resistant to the growth inhibitory effects of 1, 25-D_3. Treatment with 1, 25-D_3 increased IGFBP-3 mRNA expression in both cell lines but increases in intracellular IGFBP-3 protein and its secretion were observed only in MCF-7. 1, 25-D_3-induced apoptosis was not associated with activation of any caspase but PARP-1 cleavage was detected in parental cells. IGFBP-3 treatment alone produced cleavage of caspases 7, 8, and 9 and PARP-1 in MCF-7 cells. IGFBP-3 failed to activate caspases in MCF-7/VD^R cells; however PARP-1 cleavage was detected. 1, 25-D_3 treatment inhibited IGF-I/Akt survival signalling in MCF-7 but not in MCF-7/VD^R cells. In contrast, IGFBP-3 treatment was effective in inhibiting IGF-I/Akt pathways in both breast cancer lines. These results suggest a role for IGFBP-3 in 1, 25-D_3 apoptotic signalling and that impaired secretion of IGFBP-3 may be involved in acquired resistance to vitamin D in breast cancer.

1. Introduction

The insulin-like growth factor I (IGF-I) system is essential for normal growth and development. IGF-I is known to modulate control by insulin of normal carbohydrate and lipid metabolism. In addition, IGF-I has been reported to play a role in several pathological conditions. Interaction with the IGF binding proteins (IGFBPs) has been shown to both enhance and attenuate actions of IGF-I [1]. In addition, the IGFBPs are known to possess intrinsic growth regulatory activity, independent of their interactions with IGF-I. Insulin-like growth factor I (IGF-I) is implicated in breast cancer development and has been shown to rescue breast cancer cells from apoptosis induced by a range of chemotherapeutic agents [2]. Cellular responsiveness to IGF-I growth stimulation depends on the expression and activity of the signal transducing IGF-I receptor (IGF-IR) and a family of structurally related insulin-like growth factor binding proteins (IGFBP-1 to IGFBP-7). The major carrier of IGF-I in the circulation is IGFBP-3, which has been shown to inhibit cell growth and induce apoptosis in several cancer cell lines [3]. IGFBP-3 has been shown to regulate cell growth through both IGF-IR-dependent and -independent mechanisms (reviewed in [4]). The latter may involve signalling through an alternative cell surface receptor [5] or may involve direct nuclear actions by IGFBP-3 [6].

A number of factors with potent growth-inhibitory and apoptosis-inducing effects have been shown to induce the expression and secretion of IGFBP-3 in breast cancer cell lines, including 1, 25-dihydroxyvitamin D_3 (1, 25-D_3), the active metabolite of vitamin D_3 which has been shown to inhibit breast cancer cell growth [7]. This finding suggests that IGFBP-3 may mediate or facilitate the inhibitory effects of 1, 25-D_3. The aim of our study was to evaluate the role of IGFBP-3 in 1, 25-D_3-induced apoptosis in breast cancer cells. To this end, IGFBP-3 expression and secretion were investigated in parental MCF-7 breast cancer cells and the 1, 25-D_3-resistant cell line MCF-7/VD^R. This cell line is a vitamin-D-resistant clone of MCF-7 cells, which was developed by

incubation of parental cells with a low concentration of 1, 25-D_3, separating out the viable (resistant) cells and repeating this procedure with increasing concentrations of 1, 25-D_3 [8]. This cell line contains fully functional VDR, although in a lower number than seen with the parental MCF-7 cells. The regulation of the 24-hydroxylase enzyme appeared to be intact and no differences with regard to growth rate and morphological appearance between parental and resistant clone were observed. The MCF-7/VD^R cell line thus provides a valuable tool for identifying the exact mechanism of action of vitamin D and the development of vitamin D resistance.

2. Materials and Methods

2.1. Cell Culture and Reagent. MCF-7 human breast cancer cells were obtained from the European tissue culture collection and used between passages 5 and 20. Vitamin-D-resistant MCF-7/VD^R cells were obtained as a gift from Dr. Mork Hansen [8]. Both parental and resistant cells were grown in RPMI 1640 supplemented with 2 mM of glutamine, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 2% of foetal bovine serum (FBS). 1, 25-D_3 (Sigma UK) was used at a concentration of 100 nM and IGFBP-3 (R&D Systems) up to 100 nM.

2.2. Viability Assay. MCF-7 and MCF-7/VD^R cells were seeded into 24 well plates at a density of 1×10^4 cells/well. After 24 h, cells were treated with reagents or vehicle for up to six days. At the end of the incubation period, medium was removed and cells were incubated with neutral red solution (40 μg/mL in phenol red-free medium) for 2 h at 37°C. Following washing, fixation, and solubilisation, absorbance at 550 nm was determined.

2.3. Western Blot Analysis. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1X PBS. Equal amounts of protein (30 μg per lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in 0.05% Tween-20/TBS and then incubated with the primary antibody of interest overnight. Membranes were then incubated with the appropriate secondary horseradish-peroxidase-conjugated antibody. Bands were visualised using the enhanced chemiluminescence Western blotting detection system (ECL, Amersham). Anticleaved caspases 7, 8, and 9 and Poly [ADP-ribose] polymerase 1 (PARP-1) and antitotal caspases 7, 8, and 9, phospho-Akt, and PARP-1 antibodies were purchased from Cell Signalling. Anti-β-actin (Sigma Aldrich) was used as a loading control.

2.4. RNA Isolation and cDNA Synthesis. Total RNA from cells was extracted by using the PureLink RNA Mini-kit (Invitrogen). The quantity and the quality of RNA extracted was estimated by Nano-drop Spectrophotometer. For the reverse transcription, 2 μg of RNA was resuspended in 10 μL of nuclease free water with 2 μL random hexamer (50 μg) and was incubated at 70°C for 5 min. Then, the samples were resuspended with 13 μL of Master Mix (5 μL RT 5X Buffer, 2.5 μL of dNTP 10 mM, 0.5 μL Rnase OUT 40 U/μL, 0.5 μL of Reverse Transcriptase (MMLV, Promega), and 3.5 μL of Nuclease Free Water). This mix was run for 1 h at 42°C, 5 min at 95°C, and 5 min at 4°C. The cDNA was stored at −20°C.

2.5. RT-PCR Analysis of IGFBP-3 mRNA. The primers used to amplify IGFBP-3 and 28S rRNA were IGFBP-3 forward (GAAGGGCGACACTGCTTTTTC), IGFBP-3 reverse (CCAGCTCCAGGAAATGCTAG), 28S forward (GTTCACCCACTAATAGGGAAC), and 28S reverse (GGATTCTGACTTAGAGGCGTT). PCR was carried out in a total volume of 50 μL containing 3 μL of cDNA sample and 10 μM sense and antisense primers. The RT-PCR exponential phase was determined in 28 to 33 cycles to allow quantitative comparisons. IGFBP-3 cDNA was amplified at 94°C for 2 minutes followed by 33 cycles at 94°C for 45 seconds, 63°C for 45 sec, and 72°C for 1 minute. 28S cDNA was amplified at 94°C for 2 minutes followed by 28 cycles at 94°C for 45 seconds, 58°C for 45 sec, and 72°C for 1 minute. Final extension was performed at 72°C for 5 min. Amplification products (8 μL) were resolved in 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

2.6. Detection of IGFBP-3 Secretion in Medium by ELISA Assay. IGFBP-3 protein level in each 200 μL of medium and 100 μg of cell extract was determined using a human IGFBP-3 ELISA kit (RayBioTech, USA) according to the manufacturer's protocol.

2.7. Antibody Specific Array. Mitogen-activated protein kinases (MAPK) protein phosphorylation was measured in each 300 μg of cell extracts using Human Phospho-MAPK Array Kit according to the manufacturer (Proteome Profiler; R&D Systems). Briefly, antibody array membranes were incubated with protein lysates and then incubated with antibody array biotinylated antibody. Finally the membranes were incubated with streptavidin HRP-conjugated antibody. Immunoreactivity was visualized using a chemiluminescent substrate. Densitometric analysis was performed using GS-800 Calibrated Densitometer (Bio-Rad, UK).

2.8. Statistics. Data are reported as mean ± SD and analyzed with one-way ANOVA followed by the Bonferroni posttest for multiple comparisons using GraphPad Prism version 4.0. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Effects of 1, 25-D_3 on Growth and IGFBP-3 Expression in Parental MCF-7 and Resistant MCF-7/VD^R Cells. MCF-7 and MCF-7/VD^R cells were treated with increasing concentrations of 1, 25-D_3 for up to 6 days. Cell viability was examined by neutral red dye assay (Figure 1(a)). Whilst 1, 25-D_3 significantly decreased viability of MCF-7 cells, it had no significant effect on MCF-7/VD^R cell viability (Figure 1(a)). To determine effects on IGFBP-3 mRNA expression, MCF-7 and MCF-7/VD^R cells were treated with 100 nM 1, 25-D_3 for up to 5 days. Whole RNA was extracted from the cells

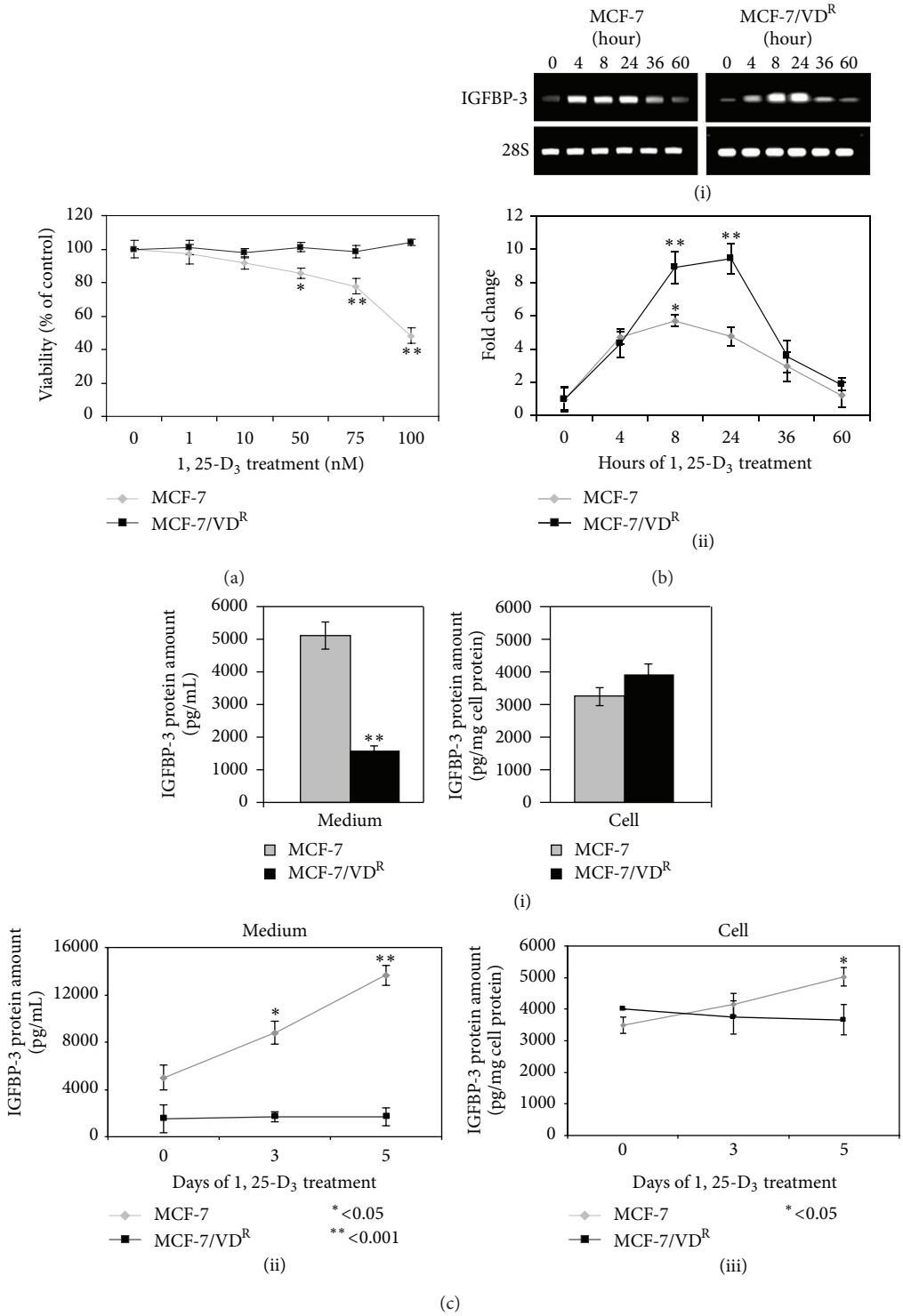

Figure 1: Effect of 1, 25-D_3 on MCF-7 and MCF-7/VD^R cell viability and IGFBP-3 expression. (a) MCF-7 and MCF-7/VD^R cells were treated with increasing concentrations of 1, 25-D_3 (up to 100 nM) or 0.1% ethanol vehicle as a control for 6 days. Cell viability was determined by neutral red assay. Means of 3 separate experiments are shown. $^*P < 0.05$ and $^{**}P < 0.001$ are statistically significant compared to the control. (b) (i) MCF-7 and MCF-7/VD^R cells were treated with 100 nM 1, 25-D_3 for up to 60 hours. IGFBP-3 mRNA expression was measured by RT-PCR. 28S mRNA expression was used as house-keeping gene. Nontreated cells were used as controls. (ii) Densitometric analysis of of IGFBP-3 mRNA expression. Data shown means of three separate experiments. (c) Intracellular IGFBP-3 levels and secretion into medium was determined by ELISA in MCF-7 and MCF-7/VD^R cells. (i) IGFBP-3 expression and secretion into the medium in untreated MCF-7 and MCF-7/VD^R cells. (ii) IGFBP-3 secretion into the medium in MCF-7 and MCF-7/VD^R cells treated with 100 nM of 1, 25-D_3 for up to 5 days quantitated by ELISA. (iii) The amount of intracellular IGFBP-3 production by MCF-7 and MCF-7/VD^R cells treated with 100 nM of 1, 25-D_3 was measured at day 0, 3, and 5 by ELISA. Means of 3 separate experiments are shown. $^*P < 0.05$ and $^{**}P < 0.001$ are statistically significant compared to the control.

at different times of treatment and IGFBP-3 expression was examined by RT-PCR (Figure 1(b)). In both MCF-7 and MCF-7/VD^R cells, 1, 25-D$_3$ treatment enhanced IGFBP-3 mRNA expression indicating that 1, 25-D$_3$ was effective in inducing IGFBP-3 mRNA expression irrespective of the observed resistance in MCF-7/VD^R cells to 1, 25-D$_3$-induced apoptosis (Figure 1(b)).

Next, we examined effects of 1, 25-D$_3$ on IGFBP-3 at the level of protein expression and secretion in MCF-7 versus MCF-7/VD^R cells. Cells were treated with 100 nM 1, 25-D$_3$ for up to 5 days. The amount of IGFBP-3 protein present in the cell or secreted into the medium was assessed by ELISA. The basal level of intracellular IGFBP-3 protein expression was found to be similar in both cell lines ($P > 0.05$); however, the amount of IGFBP-3 protein in medium conditioned by parental MCF-7 cells was significantly higher than for the resistant cell line ($P < 0.001$), indicating a reduced secretion of IGFBP-3 by the MCF-7/VD^R cells (Figure 1(c)). In addition, 1, 25-D$_3$ treatment induced IGFBP-3 protein expression and secretion in MCF-7 but not in MCF-7/VD^R cells ($P < 0.05$ and $P < 0.001$, resp.). Taken together, these results showed that impaired secretion but not transcriptional regulation of IGFBP-3 is associated with resistance of MCF-7/VD^R to 1, 25-D$_3$.

3.2. 1, 25-D$_3$- and IGFBP-3-Induced Apoptosis in MCF-7 and MCF-7/VD^R Cells. To compare characteristics of 1, 25-D$_3$- and IGFBP-3-induced apoptosis, parental and MCF-7/VD^R cells were treated for 5 days with 100 nM 1, 25-D$_3$ or 100 nM IGFBP-3. Activation of caspases 7, 8, and 9 was monitored by detection of cleaved (active) caspase fragments by immunoblotting. In addition, PARP-1 cleavage was examined and β-actin was used as a house-keeping protein (Figure 2). 1, 25-D$_3$ treatment did not lead to activation of any caspase but induced PARP-1 cleavage in parental MCF-7 but not in MCF-7/VD^R cells. In contrast, IGFBP-3 treatment produced cleavage of caspases 7, 8, and 9 and PARP-1 in MCF-7 cells. IGFPB-3 failed to activate caspases in MCF-7/VD^R cells; however PARP-1 cleavage was detected indicating an alternative pathway by which the protein induces apoptosis in these vitamin-D-resistant cells.

3.3. Effect of 1, 25-D$_3$ and IGFBP-3 on IGF-I/Akt Survival Signalling in MCF-7 and MCF-7/VD^R Cells. While parental MCF-7 cells do not express detectable IGF-I [9], the cells respond to the mitogenic and antiapoptotic effects of exogenous IGF-I and previous experiments have demonstrated that vitamin D treatment can attenuate the survival effect of IGF-I in parental MCF-7 cells [10]. To compare effects on IGF-I-mediated cell survival, MCF-7 and MCF-7/VD^R cells were treated with 100 nM 1, 25-D$_3$ and 30 nM IGF-I, alone or in combination in serum-free medium and cell viability was examined by neutral red dye assay. Cells were also cultured in medium supplemented with 2% foetal bovine serum as a control. For both cell lines serum deprivation induced up to 70–80% of cell death compared to cells cultured in the presence of serum ($P < 0.001$) and addition of IGF-I to serum-free medium rescued cell viability ($P > 0.05$ compared to control). 1, 25-D$_3$ treatment attenuated prosurvival effects of IGF-I in parental but not in resistant MCF-7/VD^R cells (Figure 3(a)). Failure of 1, 25-D$_3$ to modulate IGF-I survival signalling in resistant cells could be due to differential regulation of IGF-I bioavailability by IGFBPs such as IGFBP-3, which is not secreted by these cells.

We next compared 1, 25-D$_3$ and IGFBP-3 treatment on MAPK and Akt activation in parental and resistant cells since it is well documented that IGF-I/MAPK and IGF-I/Akt signalling plays a crucial role in proliferation and survival of breast cancer cells. Cells were treated for 5 days with 100 nM 1, 25-D$_3$ and 30 nM IGF-I, alone or in combination in serum-free medium. Cells were collected and isolated proteins were analysed on human phospho-MAPK antibody array. With respect to Akt phosphorylation, 1, 25-D$_3$ attenuated the positive effect of IGF-I on activation of Akt in MCF-7 cells but failed to do so in MCF-7/VD^R cells (Figure 3(b)). No significant differences in activation of ERK, JNK, and p38 were detected between the two cell lines with these treatments (data not shown). Differential effects of 1, 25-D$_3$ in parental and resistant cells on IGF-I-stimulated Akt activation were confirmed by immunoblotting. In contrast, treatment with IGFBP-3 reduced IGF-I-stimulated Akt phosphorylation in both cell lines (Figures 4(a) and 4(b)).

4. Discussion

The IGFBPs are secreted proteins, which bind to IGFs with high affinity. The IGFBP family has 7 distinct subgroups, IGFBP-1 through 7, and their production is tissue-type specific. Approximately 98% of IGF-1 is always bound to one of these binding proteins. The IGFBPs help to lengthen the halflife of circulating IGFs in all tissues and enhance or attenuate IGF signaling depending on their physiological context. IGFBP-3 is the most abundant of the family and accounts for 80% of all IGF binding [11]. IGFBP-3 is known to control IGF-I signalling leading to differential regulation of cell growth and apoptosis [12, 13]. A number of growth factors and hormones, including 1, 25-D$_3$, have been shown to induce the expression of IGFBP-3 in breast cancer cell lines [7]. Comparative expression profiling of human IGFBP genes in different cancer cells demonstrated that IGFBP-1, -3 and -5 are primary 1, 25-D$_3$ target genes [14]. In breast cancer, it was shown that 1, 25-D$_3$ causes cyclical IGFBP-3 mRNA accumulation with a periodicity of 60 min [15]. Accordingly, VDR also showed cyclical ligand-dependent association with the chromatin regions of its VDREs. Interestingly, HDAC4 and HDAC6 proteins, which are upregulated in a cyclical fashion in response to 1, 25-D$_3$, show cyclical VDR ligand-induced association with VDRE regions of the IGFBP-3 gene. Available evidence indicates that IGFBP-4 and 6 are not primary 1, 25-D$_3$ target genes [14]. IGFBP-5 can colocalize with VDR in the nucleus and modulate vitamin D responses in osteoblasts [16]. IGFBP-6 has also been reported to interact with VDR in the bone [17].

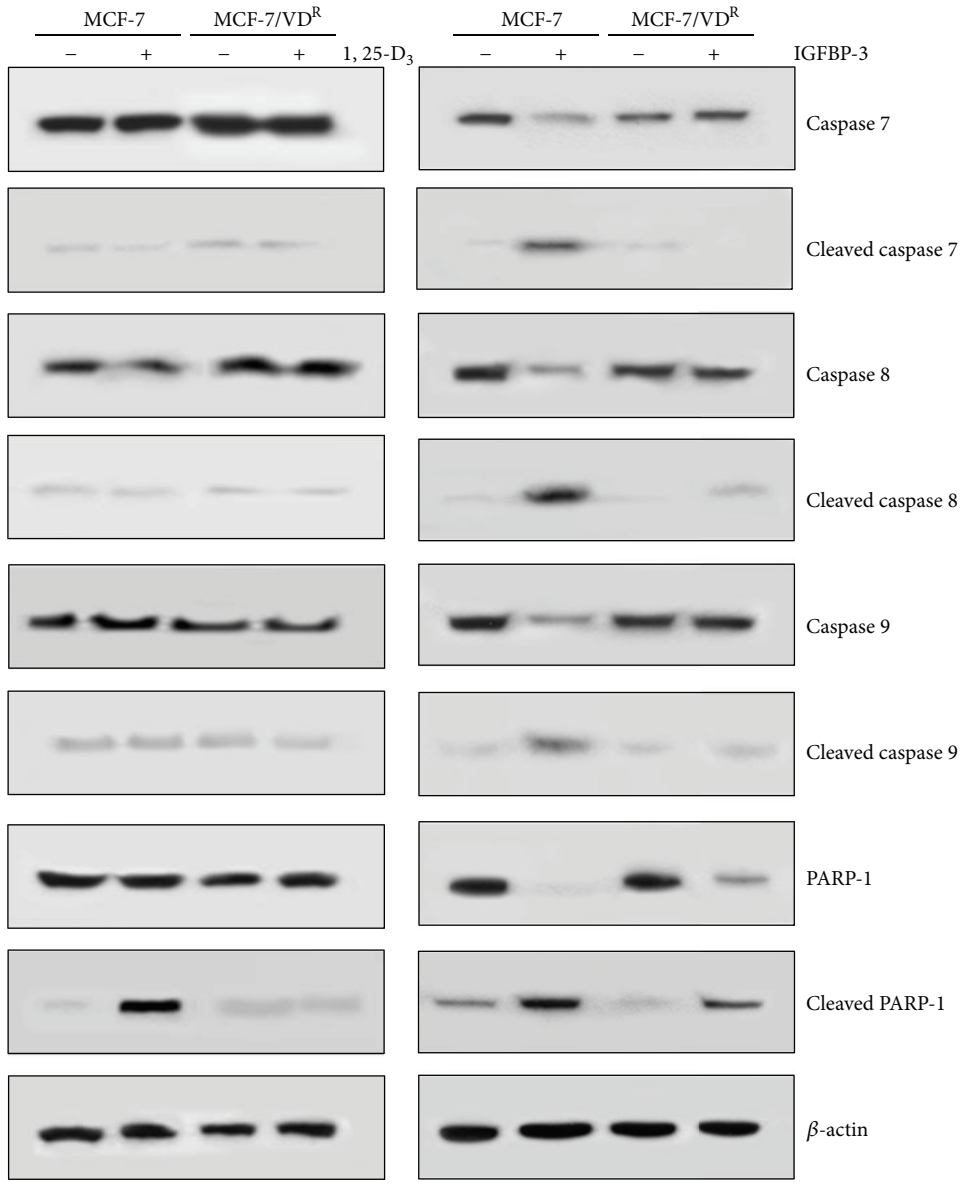

Figure 2: Caspase activation in response to 1, 25-D_3 and IGFBP-3. MCF-7 and MCF-7/VD^R cells were treated with 100 nM 1, 25-D_3 or 0.1% ethanol as vehicle control for 5 days (left hand panel) or 100 nM IGFBP-3 for 3 days. Control cells received an equal volume of PBS diluent (right-hand panel). Whole cell extracts were prepared and analysed by immunoblotting using specific antibodies of interest and β-actin was used as a loading control. Data shown are representative of three identical experiments.

Although there is an increasing body of evidence that 1, 25-D_3 exerts potent regulatory effects on breast cancer cell growth, differentiation, and apoptosis [18], the mechanisms involved are not fully understood. Our results demonstrate that 1, 25-D_3 treatment leads to an increase in IGFBP-3 mRNA in both sensitive and resistant MCF-7 cell lines, suggesting that the resistance to 1, 25-D_3 is not due to impairment in IGFBP-3 gene expression at the mRNA level. This result was not surprising because the MCF-7/VD^R cells have been reported to express a functional Vitamin D receptor [8]. We next determined if there was a difference between sensitive and resistant cells at the level of IGFBP-3 protein. Whilst there was no difference in basal intracellular IGFBP-3 protein expression, the level of IGFBP-3 in conditioned medium from resistant cells was significantly lower than medium from parental MCF-7 cells. Furthermore, we detected a clear impairment of increased expression and secretion of this protein in MCF-7/VD^R cells in response to 1, 25-D_3 treatment compared to parental cells, suggesting that effective secretion of this protein facilitates 1, 25-D_3 responsiveness. A functional role of secreted versus nonsecreted IGFBP-3 is an interesting issue in the literature. One study demonstrated that nuclear translocation of IGFBP-3 and induction of apoptosis in parental MCF-7 cells require IGFBP-3 secretion and reuptake

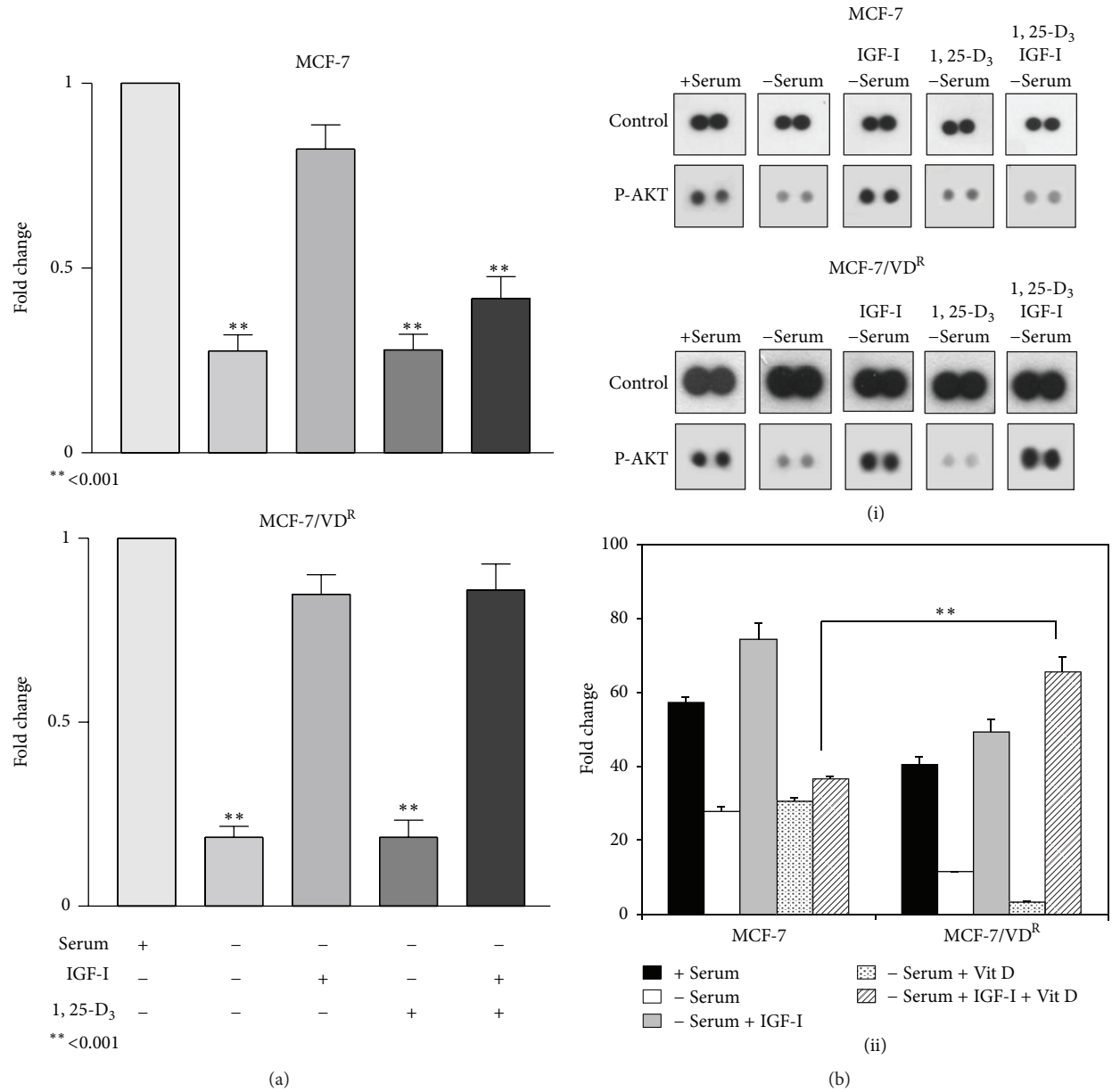

FIGURE 3: Modulation of IGF-induced Akt phosphorylation in response to 1, 25-D_3 treatment in MCF-7 and MCF-7/VD^R cells. (a) MCF-7 and MCF-7/VD^R cells were treated with 100 nM 1, 25-D_3 or 30 nM IGF-I, alone or in combination in serum-free medium. Cells were also cultured in medium supplemented with 2% serum as a control. After 6 days of treatment, cell viability was estimated by neutral red assay. $^*P < 0.05$ and $^{**}P < 0.001$ are statistically significant compared to control. Means of 3 separated experiments are shown ($n = 12$). (b) (i) MCF-7 and MCF-7/VD^R cells were treated with 100 nM 1, 25-D_3 or 30 nM IGF-I, alone or in combination, in serum-free medium. After 5 days of treatment, whole cell extracts were prepared and analysed on a phospho-MAPK antibody array (R&D Systems, UK) following manufacturer's instruction. (ii) Densitometric analysis of Akt phosphorylation. Data shown are means of 3 replicates, significantly different from parental cells. $^{**}P < 0.001$.

[19]. In contrast, Battacharyya and colleagues [20] reported that secreted and non-secreted IGFBP-3 may be functionally equivalent in induction of apoptosis in prostate cancer cells. Several studies have indicated that structural modifications such as glycosylation [21] and phosphorylation [22] can affect IGFBP-3 binding activity. However an ELISA approach, as used in our study, was unable to detect any such differences in secreted IGFBP-3 from MCF-7 cells.

It has been previously reported that 1, 25-D_3 and IGFBP-3 induce MCF-7 cell death [23, 24]. Available evidence suggests differences in the characteristics of 1, 25-D_3- and IGFBP-3-induced apoptosis in MCF-7 cells. It was previously reported

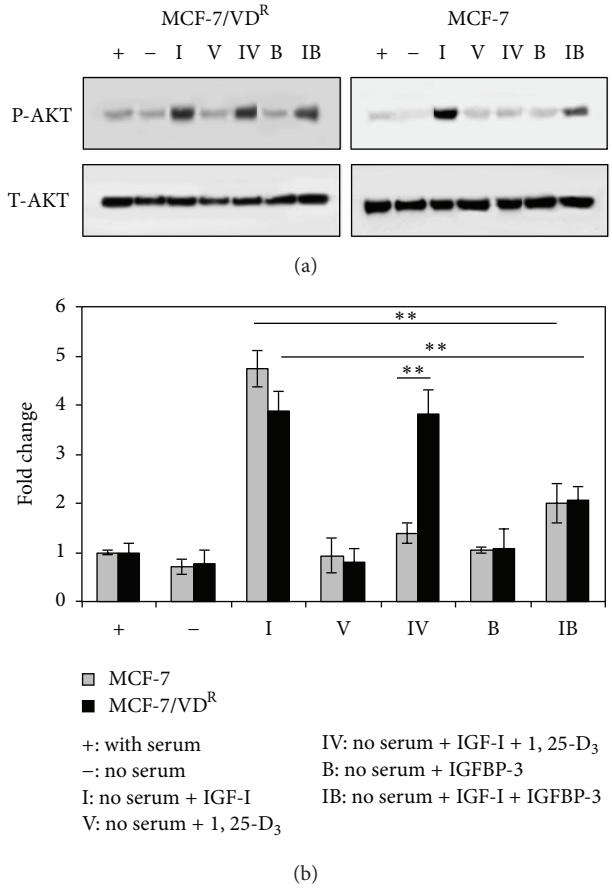

Figure 4: Differential modulation of IGF-induced Akt phosphorylation in response to 1, 25-D_3 and IGFBP-3 treatment in MCF-7 and MCF-7/VD^R cells. (a) MCF-7 and MCF-7/VD^R cells were treated with 100 nM 1, 25-D_3 or 30 nM IGF-I, alone or in combination, in serum-free medium. Cells were also treated with 100 nM IGFBP-3 alone or in combination with 30 nM IGF-I in serum-free medium. After 5 days of treatment, whole cell extracts were prepared and analysed by immunoblotting for total-Akt (T-Akt) and phospho-Akt (P-Akt). (b) Densitometric analysis of immunoblots was performed using GS-800 Calibrated Densitometer (Bio-Rad UK). Data shown are representative of three identical experiments. Means of 3 separated experiments are shown. $^*P < 0.05$ and $^{**}P < 0.001$ are statistically significant.

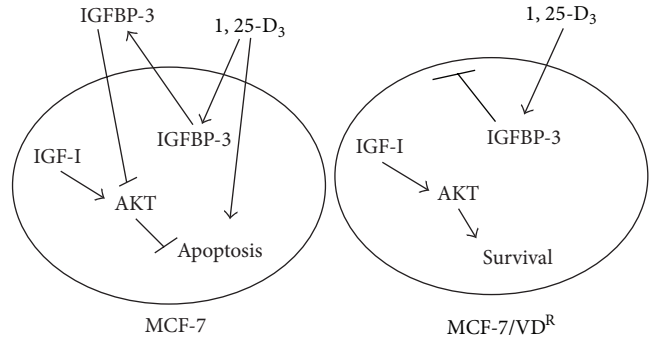

Figure 5: Proposed interaction between 1, 25-D_3 and IGFBP-3 in MCF-7 and MCF-7/VD^R cells. In parental cells stimulation by 1, 25-D_3 of IGFBP-3 secretion attenuates IGF-1-induced activation of Akt, leading to apoptosis. In addition, 1, 25-D_3 may initiate caspase-independent pathways contributing to cell death in parental cells. In resistant cells, failure of IGFBP-3 secretion is associated with activation of the IGF-I/Akt pathway, leading to cell survival.

that 1, 25-D_3 induced apoptosis in a caspase-independent manner in MCF-7 cells [25] and this is confirmed in our present study. Whilst we found that 1, 25-D_3 treatment did not induce activation of caspases 7, 8, and 9 in either MCF-7 or MCF-7/VD^R cell line, PARP-1 cleavage was detected in parental but not resistant cells. Indeed, it has previously been demonstrated that PARP-1 cleavage associated with 1, 25-D_3-induced apoptosis could involve other proteinases such as calpains [26]. In contrast, we found that exogenous IGFBP-3-stimulated activation of caspases 7, 8 and 9 in parental MCF-7 but not in MCF-7/VD^R cells. However PARP-1 cleavage was detected in response to IGFBP-3 treatment in both cell lines suggesting that IGFBP-3 produces PARP-1 cleavage in a caspase-independent manner in MCF-7/VD^R cells. PARP-1 processing leading to activation of nucleases and DNA fragmentation appears as a key point in the execution phase of apoptosis. In support of our findings other reports have demonstrated PARP-1 processing in the absence of any caspase activation suggesting the role of other proteases in this process [27–29]. The ability of IGFBP-3 to induce apoptosis by both caspase-dependent and caspase-independent mechanisms suggests that this protein could act through two different signalling pathways. This observation also suggests that biochemical properties of endogenous and secreted IGFBP-3 may differ from exogenous protein.

A number of studies have indicated IGF-I-dependent and -independent mechanisms by which IGFBP-3 induces apoptosis. By limiting IGF-I bioavailability, IGFBP-3 controls signal transduction through the IGF-I receptor, including survival signalling and induction of cell death. Exogenous IGFBP-3 also appears to exert IGF-I-independent effects, activating apoptosis via novel or death receptor pathways [9, 30, 31]. In contrast, other studies have shown that IGFBP-3 modulates RXR/Nur77 signalling in the nucleus, thereby inducing apoptosis in a mitochondria-dependent manner [32]. We found that RXR-α is expressed only in parental MCF-7 but not in MCF-7/VD^R cells (unpublished observations) suggesting that a RXR/Nur pathway does not exist in these cells and this is supported by absence of caspase 9 activation in response to IGFBP-3.

It is well documented that Akt activation plays a crucial role in antiapoptotic actions of IGF-I in breast cancer cells and our initial experiments clearly demonstrated that 1, 25-D_3 treatment attenuated the survival effect of IGF-I in parental cell line but not in resistant MCF-7/VD^R cells. Our results using MAPK/Akt antibody array analysis demonstrated that 1, 25-D_3 attenuated IGF-I-induced Akt phosphorylation in MCF-7 cells but failed to do so in MCF-7/VD^R cells, suggesting that failure to modulate IGF-I/Akt survival signalling could contribute to the resistance of this cell line to 1, 25-D_3. In contrast, induction of apoptosis by exogenous IGFBP-3 in both 1, 25-D_3-sensitive and -resistant cells was associated with

inhibition the IGF-I/Akt pathway (Figure 5). In this regard the ability of IGFBP-3 to downregulate Akt activity in Her2 overexpressing MCF-7 cells has been previously reported [33].

5. Conclusion

Taken together our results suggest a role for IGFBP-3 in 1, 25-D_3 apoptotic signalling and impaired secretion of IGFBP-3 may be involved in acquired resistance to vitamin D in breast cancer cells. In addition, regulation of IGF-I/Akt survival signalling may function as a key point of convergence that may determine breast cancer cell fate in response to 1, 25-D_3/IGFBP-3.

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Research Article

Mitochondrial Complex I Inhibitors and Forced Oxidative Phosphorylation Synergize in Inducing Cancer Cell Death

Roberta Palorini,[1,2] Tiziana Simonetto,[1] Claudia Cirulli,[1,2] and Ferdinando Chiaradonna[1,2]

[1] *Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy*
[2] *SysBio Centre of Systems Biology, Piazza della Scienza 2, 20126 Milan, Italy*

Correspondence should be addressed to Ferdinando Chiaradonna; ferdinando.chiaradonna@unimib.it

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Cancer cells generally rely mostly on glycolysis rather than oxidative phosphorylation (OXPHOS) for ATP production. In fact, they are particularly sensitive to glycolysis inhibition and glucose depletion. On the other hand mitochondrial dysfunctions, involved in the onset of the Warburg effect, are sometimes also associated with the resistance to apoptosis that characterizes cancer cells. Therefore, combined treatments targeting both glycolysis and mitochondria function, exploiting peculiar tumor features, might be lethal for cancer cells. In this study, we show that glucose deprivation and mitochondrial Complex I inhibitors synergize in inducing cancer cell death. In particular, our results reveal that low doses of Complex I inhibitors, ineffective on immortalized cells and in high glucose growth, become specifically cytotoxic on cancer cells deprived of glucose. Importantly, the cytotoxic effect of the inhibitors on cancer cells is strongly enhanced by forskolin, a PKA pathway activator, that we have previously shown to stimulate OXPHOS. Taken together, we demonstrate that induction in cancer cells of a switch from a glycolytic to a more respirative metabolism, obtained by glucose depletion or mitochondrial activity stimulation, strongly increases their sensitivity to low doses of mitochondrial Complex I inhibitors. Our findings might be a valuable approach to eradicate cancer cells.

1. Introduction

As indicated by Otto Warburg many years ago and now accepted as a hallmark of cellular transformation, cancer cells entirely reprogram their metabolism to sustain hyperproliferation and growth also in particular environmental conditions [1]. In particular, differently from normal cells, cancer cells rely mostly on glycolysis rather than oxidative phosphorylation (OXPHOS) for ATP production [2, 3]. Tumor environment, oncogenes, and tumor suppressor mutations have an important role in this energetic shift to aerobic glycolysis [4, 5]. Another important feature of metabolic reprogramming of transformed cells is their reduced or strongly impaired mitochondrial function [3, 6]. Despite that, mitochondria cover an important role also in cancer cells, that is, through the maintenance of mitochondrial potential and oxidative equilibrium, necessary for cell viability and apoptosis control, and for the different anabolic processes that use precursors produced in this organelle such as lipid, amino acids, and nucleotides synthesis. Thus, different therapeutic approaches have been addressed to cancer cell mitochondria. There is a series of compounds targeting mitochondria, named mitocans, that are being tested as anticancer drugs. They usually lead to cancer cell death by inducing mitochondria destabilization with a consequent increase of reactive oxigen species (ROS) and activation of apoptotic signals [7, 8]. Different classes of mitocans exist and can be classified into eight groups, more specifically hexokinase inhibitors, Bcl-2 homology-3 (BH3) mimetics, thiol redox inhibitors, drugs targeting the voltage-dependent anionic channel (VDAC) or the adenine nucleotide translocator (ANT), agents interfering with the electron transport chain (ETC), lipophilic cations targeting the inner membrane, agents interfering with the mitochondrial DNA, and drugs acting on not well-defined sites [8]. Among the compounds acting on the ETC, vitamin E analogues that in particular target Complex II have been

tested as anticancer agents [9]. Complex I inhibitors have shown anticancer properties as well, for example the acetogenins, such as rollinistatin and bullatacin, and also rotenone itself, which exhibits antitumor activity in animal models [10].

On the other hand, cancer cells for their peculiar metabolism are particularly sensitive to treatments inhibiting glycolysis and to glucose deprivation [11, 12], since in both circumstances they lose hyperproliferative ability and ultimately die [12–15]. Therefore, combined treatment targeting both glycolysis and mitochondria, exploiting peculiar tumor features, may be lethal for cancer cells. In this regard it has been shown that cancer cells, like osteosarcoma cells, treated with ETC inhibitors, are induced to switch over to glycolysis becoming hypersensitive to the glycolytic inhibitors [16]. Equally, it has been shown that inhibition of glucose metabolism, for example, by using 2-deoxyglucose (2-DG), can make tumor cells more dependent on OXPHOS and therefore more sensitive to treatment with ETC inhibitors [17]. However, glycolytic inhibitors, like 2-DG, could be potentially toxic for tissues like the brain, retinae, and testis that use glucose as the main energy source. In addition, they are also not very potent and must be used at high concentrations [11].

In a previous study it has been shown that treatment of cancer cells with dichloroacetate (DCA), a TCA cycle inducer, is able to redirect their metabolism from glycolysis to oxidative phosphorylation and hence to lead them towards apoptosis [18]. Therefore, it has been supposed that induction of a reversion of the Warburg effect coupled to a treatment able to interfere with mitochondrial activity could specifically kill cancer cells. Recently we have shown that exogenous activation of PKA pathway can improve several mitochondrial parameters, leading to a Warburg effect reversion, in K-ras cancer cells, where the Protein Kinase A (PKA) pathway is generally deregulated [19]. In fact, cancer cells treated with forskolin (FSK), an activator of adenylate cyclase [20], show an increase of Complex I activity, an increase of mitochondrial ATP production, a decrease of ROS generation, and an increase of mitochondria interconnections, that may lead to survival under glucose depletion [15].

Since nutrient deprivation widely exists in solid tumors because of the poor blood supply [21, 22], we decided to study the effects on cancer cells of glucose depletion, mimicking physiological tumor condition, instead of glycolysis inhibitors, combined with treatments with OXPHOS Complex I inhibitors. As results we demonstrate that in low glucose availability different cancer cell lines, in a way dependent on their glycolytic metabolism, become sensitive to short treatment with low doses of Complex I inhibitors as compared to optimal glucose condition. In fact we observe an increased cell death. Interestingly, in such a glucose-depleted condition, we also find evidence that stimulation of mitochondrial activity by FSK can further sensitize cancer cells to Complex I inhibitors by enhancing cancer cell death. Altogether our findings indicate that stimulation of respiratory chain activity, in low glucose availability, makes glycolytic cancer cells more sensitive to OXPHOS inhibitors.

2. Material and Methods

2.1. Cell Cultures. Breast cancer cells MDA-MB-231, mouse fibroblasts NIH3T3 Normal and Transformed, pancreatic cancer cells MIA PaCa-2, and lung cancer cells A549 [15] were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (complete medium), supplemented with 5–10% fetal bovine serum (human cells) or 10% newborn calf serum (mouse cells). For the experiments cells were plated in complete growth medium. After 16 hours cells were washed twice with phosphate buffer saline (PBS) and incubated in growth medium (time 0) without glucose and sodium pyruvate, supplemented with 25 or 1 mM glucose. Treatments and analyses were performed at 48 hours (MDA-MB-231 and A549) or 72 hours (NIH3T3 and MIA PaCa-2) after time 0. All reagents for media were purchased from Life Technologies (Carlsbad, CA, USA).

2.2. Treatments. Rotenone, oligomycin, and FSK were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Capsaicin and piericidin A were purchased from Vinci-Biochem (Florence, Italy).

2.3. Viability Assays. Cell viable count was performed by staining cells with Trypan Blue 0.4% (Life Technologies).

Propidium iodide (PI)/Annexin V-FITC staining was performed using Apoptosis Assay Kit from Immunological Sciences (Rome, Italy) and analyzed by FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) with CellQuest software (Becton-Dickinson). Flow cytometric data were then carried out using the freely available WinMDI software.

For the evaluation of PI incorporation 5×10^5 cells were harvested and stained with 5 μg/mL PI and 5 μg/mL Hoechst (Sigma-Aldrich Inc.) in PBS for 15 min at r.t. After staining, cells were mounted on a microscope slide with 50% glycerol and analyzed under a Nikon ECLIPSE 90i fluorescence microscope (Nikon, Tokyo, Japan) equipped with a b/w CCD camera (Hamamatsu-CoolSNAP, Hamamatsu Corporation, Hamamatsu City, Japan). The images were acquired using the imaging software Metamorph 7 and then visualized and processed in Image J (freely available).

2.4. Clonogenic Assay. For each sample 3×10^3 cells were plated in 100 mm dish. After ≥12 days colonies were fixed with PBS-formaldehyde 5%, stained with crystal violet 1%, and then counted.

2.5. Intracellular ATP and Mitochondrial Potential Quantification. Intracellular ATP levels were measured using CellTiter Glo luciferin-luciferase assay (Promega, Madison, WI, USA) as described in [23].

Mitochondrial potential was analyzed by staining cells with 20 nM JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, Life Technologies) for 10 minutes. After staining, flow cytometric analysis was performed acquiring FL1 (JC-1 monomers, low potential) and FL2 (JC-1 aggregates, high potential) signals. For each sample

the ratio FL2/FL1 was calculated and used to compare different samples.

2.6. D-Glucose Measurement. D-Glucose levels in culture medium were determined using a spectrophotometric assay kit (R-Biopharm, Darmstadt, Germany) as specified by manufacturer's datasheet.

2.7. Western Blot Analysis. For the analysis of cleaved Caspase 3 and Actin B expression, cells were harvested and lysed in Laemli buffer (50 mM Tris-HCl pH6.8, glycerol 6%, SDS 2%, β-mecaptoethanol 5%, bromophenol blue 0.05%). Samples were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane, which was incubated overnight with antibodies for cleaved Caspase 3 (Cell Signaling Technology Inc., Danvers, MA, USA; 1 : 1000) and Actin B (Abcam, Cambridge, UK; 1 : 1000).

2.8. Oxygen Consumption Rate (OCR) Measurement. Oxygen consumption was determined using Seahorse XF24 extracellular Flux analyzer (Seahorse Bioscience, North Billerica, MA, USA). Cells were seeded in the 24-well XF24 cell culture plate in the culture medium containing 25 or 1 mM glucose, as described above. Where indicated, cells were also treated with FSK. Culture media were exchanged for base media (unbuffered DMEM supplemented with 10 mM sodium pyruvate and 20 mM glucose for cells grown in high glucose or only 10 mM sodium pyruvate for cells grown in low glucose) 1 hour before the assay and for the duration of the experiment. Selective inhibitors were injected during the measurements to achieve final concentrations of rotenone 3 nM and piericidin A 5 nM. The baseline OCR was defined as the average of the values measured from time points 1 to 5 (0–45 min) during the experiments. Due to some variations in the absolute magnitude of OCR measurements in different experiments, the relative OCR levels were used to compare and summarize independent biological replicates. After the analysis the cells were fixed, stained with Crystal Violet, and dosed at spectrophotometer after colorant solubilization with acetic acid 10%; all OCR values obtained by the instrument were normalized on cell density.

3. Results

3.1. Complex I Inhibition by Rotenone Influences Cancer Cell Survival Depending on Initial Glucose Availability. MDA-MB-231 human breast cancer cells, like several other cancer cells, use mainly glycolysis instead of mitochondrial respiration to generate ATP and other anabolic substrates necessary for their proliferation and survival [24]. In fact, in low glucose availability, these cells show a reduced proliferation and an increase of cell death because of their inability to maximize the use of OXPHOS especially for energetic use [15]. In this scenario, we tested the ability of rotenone, an inhibitor of OXPHOS, to increase their sensitivity to glucose depletion. Rotenone is a natural compound that has been used to interfere with mitochondrial respiration, in particular with Complex I activity, and hence to reduce intracellular ATP levels especially in OXPHOS-dependent cell lines [25, 26]. In order to evaluate their ability to proliferate and survive under OXPHOS inhibition, we treated proliferating MDA-MB-231 cells, grown for 48 hours in low glucose (1 mM glucose, 4 mM glutamine) or high glucose (25 mM glucose, 4 mM glutamine), with rotenone. The treatment was executed at 48 hours of culture because, despite a comparable proliferation rate in the two different glucose concentrations (Figure 1(a)), in low glucose condition external medium analysis indicated that this carbon source was almost completely depleted at this time point (Figure 1(b)). In addition, measurement of the basal cellular oxygen consumption rate (OCR) by Seahorse XF analyzer indicated a 40% increase of cellular respiration rate in cells grown in low glucose (Figure 1(c)), suggesting that MDA-MB-213 cells, in absence of glucose as main substrate for glycolysis, partially shifted from glycolysis to mitochondrial respiration. Short treatment with a low concentration of rotenone (3 nM for 4 hours), known to be ineffective on normal cell mitochondria activity [27–29], in glucose-depleted condition induced a reduction of cell viability, as confirmed by morphological analysis (Figure 1(d), circle and floating cells) and by Trypan Blue viable cell count (Figure 1(e)). In fact, after treatment Trypan Blue-positive cells increased from 10.2% to 22.3%. This effect on cell survival was also supported by clonogenic assays (Figures 1(f) and 1(g)), which showed that treated cells, replated in high glucose condition (25 mM), formed less colonies (about 50% of reduction) as compared to untreated control. Such an assay shows that the short treatment is enough to reduce cancer cell ability to form a large colony and proliferate, suggesting that rotenone, inhibiting the alternative mitochondrial energetic route of these cancer cells upon glucose deprivation, heavily affects their viability. Rotenone outcome on mitochondrial activity was evaluated by determination of OCR, mainly due to mitochondrial respiration, mitochondrial potential, and intracellular ATP levels. In particular, 3 nM rotenone was injected by the instrument into the cells and its effect analyzed between 30 minutes and 1 hour after the injection. As shown in Figure 1(h), OCR was reduced to ~30% of the baseline rates, indicating that 3 nM rotenone is able to decrease mitochondrial respiration. Also mitochondrial potential was reduced by rotenone (Figure 1(i)), confirming the direct effect of the treatment on mitochondrial function. In the same experimental setting, rotenone induced also a ~25% decrease of the intracellular ATP levels (Figure 1(j)).

Importantly, rotenone treatment in nonlimiting glucose condition had no effect on cell survival, as confirmed by Trypan Blue viable cell count (Figure 1(k)) and clonogenic assay (Figure 1(l)). Moreover, rotenone had no effect on intracellular ATP levels (Figure 1(m)), suggesting that in high glucose availability ATP is generated essentially by glycolysis.

3.2. Under Glucose Depletion, Rotenone and FSK Cause Enhanced Mouse K-Ras-Transformed Cell Death as Compared to Immortalized Counterpart. It has been proposed that Warburg effect reversion, gained by mitochondrial reactivation, may be a promising method for promoting naturally encoded programmed cell death and hence kill cancer cells [18]. Given that, we sought to investigate whether the combination of

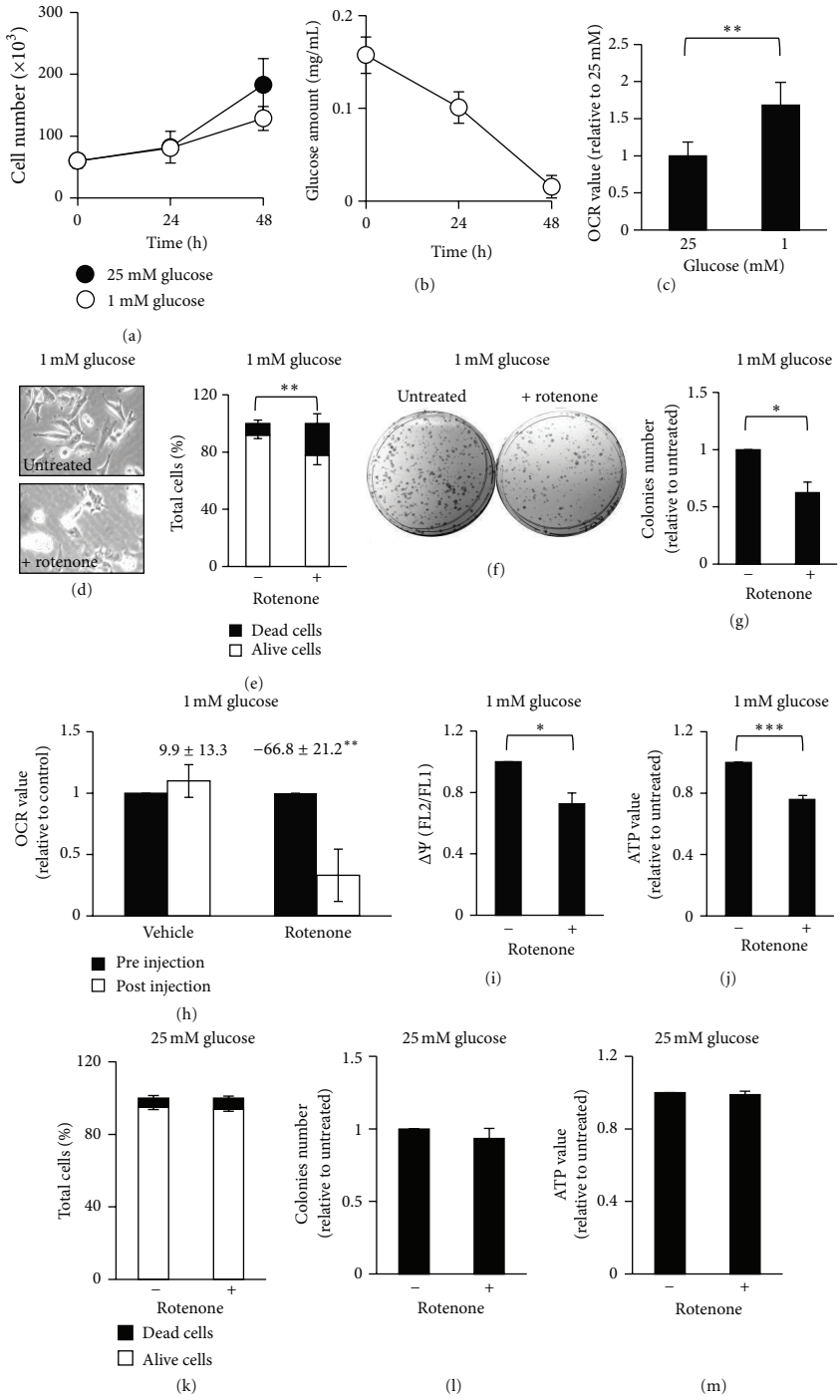

Figure 1: MDA-MB-231 cells are sensitive to rotenone in condition of glucose deprivation. (a) Proliferation curves for MDA-MB-231 cells cultured at 25 and 1 mM glucose were obtained counting cells at indicated time points. (b) Glucose amount in medium of cells cultured in 1 mM glucose was measured using enzymatic kit at indicated time points. (c) Basal OCR of MDA-MB-231 cells grown in 25 and 1 mM glucose was determined by Seahorse XF24 analyzer; data represent the average ± s.e.m. of three independent experiments (total number of samples ≥ 10), $^{**}P < 0.01$ (Student's t-test). (d)–(j) MDA-MB-231 cells cultured for 48 hours in 1 mM glucose were treated for 4 hours with 3 nM rotenone. After treatment, optical microscopy images (d) and viable cell count performed by using Trypan Blue Staining (e) were obtained for untreated (−) and treated (+) cells. After treatment 3×10^3 cells were also plated in normal growth medium for clonogenic assay and after ≥12 days colonies were stained (f) and counted (g). OCR of MDA-MB-231 cells cultured in low glucose was determined by Seahorse XF24 analyzer 30 minutes after injection of vehicle or 3 nM rotenone; the percentage of OCR variation after injection is reported (h). In untreated (−) and treated (+) cells also mitochondrial potential (ΔΨ) indicated as ratio of mean fluorescence FL2 on mean fluorescence FL1 (see Section 2) (i) and intracellular ATP levels (j) were measured. (k)–(m) Cell count (k), clonogenic assay (l), and intracellular ATP measurement (m) were performed also in cells grown for 48 hours in 25 mM glucose and treated with rotenone as above. All data represent the average of at least three independent experiments (±s.d.); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's t-test).

FSK, able to restore mitochondrial activity, and rotenone could synergistically enhance the killing of cancer cells in glucose depletion. First, we performed such an analysis on NIH3T3 mouse fibroblasts (immortalized cells, Normal), an OXPHOS-dependent cell line, and NIH3T3 mouse fibroblasts expressing an oncogenic *K-RAS* gene (Transformed) [15, 24].

The latter cellular model of transformation is suitable since it presents a transcriptional profile and different metabolic features, such as the Warburg effect, comparable to several human cancer cells harboring an oncogenic *K-RAS* gene, like, for instance, MDA-MB-231 cells [23, 24, 30, 31].

The cells grown for 72 hours in both initial glucose concentrations were incubated with rotenone and FSK alone or in combination. As shown in Figures 2(a) and 2(b), in nonlimiting glucose condition rotenone had no effect on proliferation of both cell lines, confirming that such a low rotenone concentration does not inhibit mitochondrial respiration of Normal cells (Figure 2(a)) and does not induce cell death in mouse Transformed cells (Figure 2(b)), as previously observed in MDA-MB-231 cells. On the contrary, cells grown in low glucose for 72 hours, the time point at which both cell lines have completely consumed the glucose in the culture medium [15], showed a different response to the treatments with rotenone and/or FSK (refer to Figure 2(c) for treatments schedule). Normal cells were found to be insensitive to rotenone either alone or in combination with FSK (Figure 2(d)). In contrast, Transformed cells showed 17% of cell death in basal condition, 22% upon FSK treatment, 29% upon rotenone, and 42% when the two compounds were used in combination (Figure 2(e)). These data indicate that Normal mouse cells, relying especially on mitochondrial respiration, are less responsive to low doses of rotenone as well as to the combined treatment with FSK. On the contrary, Transformed mouse cells, forced to use mitochondrial respiration by glucose deprivation or FSK treatment, become more sensitive to the Complex I inhibitor.

3.3. Under Glucose Depletion the Combined Treatment with Rotenone and FSK Leads to an Increase of MDA-MB-231 Cell Death as Compared to Rotenone Alone. Since a previous study has indicated that MDA-MB-231 cells are responsive to FSK treatment as well as mouse K-ras-transformed fibroblasts [15], we treated MDA-MB-231 cancer cells, grown in low glucose, with rotenone and FSK alone or in combination (schedule is shown in Figure 2(c)). In order to evaluate cell death upon single or combined treatments, the cells were analyzed through Trypan Blue viable count (Figure 3(a)) or PI/Annexin V staining followed by FACS analysis (Figure 3(b) and Supplementary Figure 1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2013/243876). As shown in Figures 3(a) and 3(b), both analyses indicated an increased cell death in the samples subjected to the combined treatment as compared to either untreated or rotenone-alone-treated samples. In particular, Trypan Blue Staining indicated an increase of positive cells from 22% for rotenone alone to 33.6% in presence of FSK. Similar values were observed by PI/Annexin V staining (18% rotenone versus 30% rotenone + FSK). Importantly, the combined treatment further reduced cancer cell ability to form colonies as compared to rotenone alone (Figure 3(c)). The increase of cell death was associated with a reduction of about 50% of intracellular ATP levels (Figure 3(d)) and of around 20% of mitochondrial potential (Figure 3(e)) as compared to untreated samples. To confirm a role of FSK in inducing a positive effect on mitochondrial activity, next we measured basal OCR, as previously described, in untreated or 2-to-4-hour FSK-treated cells. The interval of treatment was chosen since in our assays the cells were treated with FSK (pretreatment plus combination with OXPHOS inhibitors) for a maximum time of 5 hours. As shown in Figure 3(f), FSK-treated samples showed an increase of around 30% of OCR as compared to untreated samples, suggesting that the formers are more respirative than the latter ones. Moreover we did not observe differences in OCR values obtained in 2 and 4 hours-treated samples. Altogether these findings indicate that, upon glucose depletion, the stimulation of respiratory chain activity makes cells more sensitive to OXPHOS inhibitors.

3.4. Piericidin A and Capsaicin, Inhibitors of Mitochondrial Complex I, Show the Same Effects of Rotenone and Synergize with FSK in Inducing MDA-MB-231 Cell Death. To further confirm the role of mitochondrial inhibition in the cell death mechanism upon glucose depletion, and more specifically the role of Complex I, we used two other inhibitors of this complex, namely piericidin A and capsaicin [32]. Importantly piericidin A, differently from rotenone that at higher concentration may affect cell cycle [33, 34], does not interfere with the cell cycle execution. As shown in Figures 4(a) and 4(b), upon 2 hours of treatment, both inhibitors, as previously observed with rotenone, did not induce cell death when added to MDA-MB-231 grown in high glucose. On the contrary, their addition to glucose-depleted cells led to an increase of MDA-MB-231 cell death that was much stronger in the samples treated with piericidin A (52%) (Figure 4(c)) than with capsaicin (28%) (Figure 4(d)). Notably, combined treatment with FSK further increased the percentage of cell death that reached a value of 80% in the sample piericidin A + FSK (Figure 4(c)) and 39% in the sample capsaicin + FSK (Figure 4(d)).

Since cell viability was greatly affected by piericidin A, we evaluated also the potential of this molecule in combination with FSK in clonogenic assays (Figure 4(e)). Obtained data indicated a significant reduction of colonies number after treatment with piericidin A, reduction that was further increased upon combination with FSK as compared to untreated and FSK-treated sample (Figure 4(e)). Notably, OCR measurement indicated that piericidin A was able to decrease mitochondrial respiration (Figure 4(f)) as well as previously observed with rotenone (Figure 1(h)), confirming its effect on cellular mitochondrial respiration.

3.5. FSK Enhances Complex I Inhibitors Effect on Cancer Cell Viability by Increasing Necrotic Cell Death. Glucose deprivation has been shown to kill cells either by necrosis or through the mitochondrial pathway of apoptosis [35]. Similarly, prolonged treatment with mitochondrial OXPHOS inhibitors

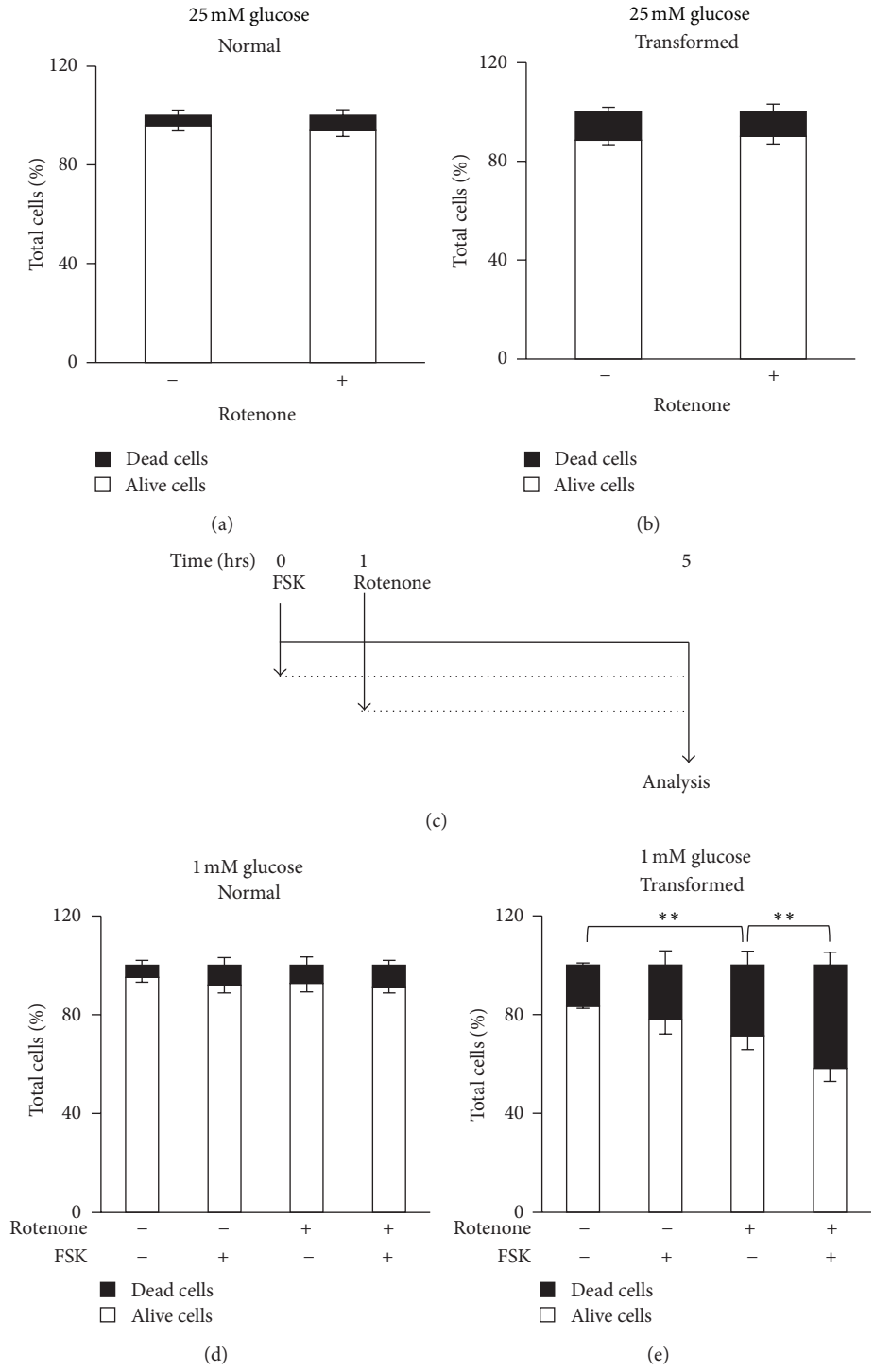

Figure 2: Low doses of rotenone do not affect Normal cells survival as compared to Transformed cells. Viable cell count using Trypan Blue was performed after treatment with rotenone at 72 hours of culture in different growth conditions. (a)-(b) NIH3T3 (a) Normal and (b) Transformed cells were cultured in 25 mM glucose and counted after 4-hour treatment with 3 nM rotenone. (c)–(e) Cells were cultured in 1 mM glucose and treated with 3 nM rotenone, 10 μM FSK, or both molecules. For the combined treatment, cells were pretreated for 1 hour with FSK and then rotenone was also added for 4 hours as represented in (c). After treatment (d) Normal and (e) Transformed cells were counted. All data represent the average of at least three independent experiments (±s.d.); $^{**}P < 0.01$ (Student's t-test).

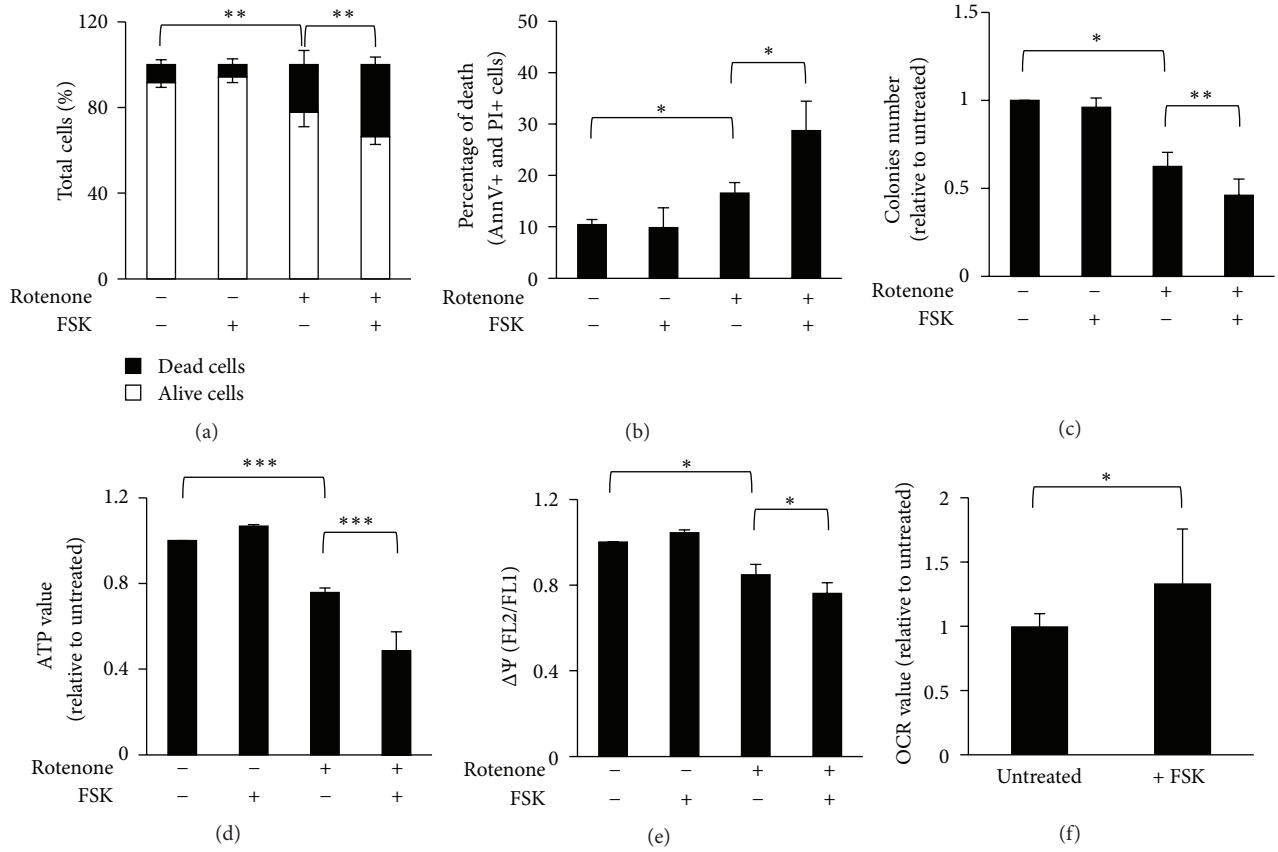

Figure 3: FSK treatment enhances the viability loss induced by rotenone alone in MDA-MB-231 cells. MDA-MB-231 cells were cultured in 1 mM glucose and treated with 3 nM rotenone, 10 μM FSK, or both molecules at 48 hours of culture. Cells were pretreated for 1 hour with FSK and then rotenone was also added for 4 hours, as shown in Figure 2(c). After treatment different parameters were investigated in untreated (–) and treated (+) cells. Viable cell count was performed using Trypan Blue (a). Percentage of cell death was evaluated after staining with PI and Annexin V-FITC (AnnV). Cells positive for one or both molecules were considered dead cells (b). After treatment 3×10^3 cells were plated in normal growth medium for clonogenic assay and after ≥12 days colonies were stained and counted as reported in the histogram (c). (d) Intracellular ATP levels and (e) mitochondrial potential were also measured. All data represent the average of at least three independent experiments (±s.d.); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's t-test). (f) Basal OCR of MDA-MB-231 cells, grown 1 mM glucose and treated or not with FSK for 2–4 hours, was determined by Seahorse XF24 analyzer. Analysis was performed in three independent experiments (total number of samples ≥ 15) and values are indicated as the average ± s.e.m.; $^{*}P < 0.05$ (Student's t-test).

also lead to necrotic cell death [36]. In order to assess whether single or combined treatments could induce necrosis or apoptosis, we performed experiments of PI incorporation followed by microscopy analysis and of Western blot. As shown in Figures 5(a) and 5(b), Complex I mitochondrial inhibitors (rotenone and piericidin A) caused an increase of PI incorporating cells as compared to untreated or FSK-treated samples. As expected from previous results, FSK treatment further enhanced the percentage of PI incorporating cells, supporting the notion that cells were experiencing a necrotic cell death process (Figures 5(a) and 5(b)). Such a cell death mechanism was confirmed by morphology analysis indicating cell detachment (data not shown) and plasma membrane damage without nuclear condensation (Figure 5(a), Hoechst staining). In addition, this cell death process was not associated with activation of Caspase 3 (Figures 5(c) and 5(d)) and poly(ADP-ribose) polymerase (PARP) (data not shown), both considered apoptotic markers, as it was seen with thapsigargin treatment for 6 hours (Figures 5(c) and 5(d)).

3.6. Mitochondrial Complex I Inhibitor Piericidin A Synergize with FSK in Inducing Death of MIA PaCa-2 and A549 Cancer Cells. To examine whether other cancer cell lines showed similar sensitivity to Complex I inhibitors alone and combined with FSK, we examined the effect of the treatments on two different human cancer cell lines with a different dependence on glucose availability. Previous data, in fact, have shown that MIA PaCa-2 (pancreatic cancer cell line) and A549 (non-small-cell lung cancer cell line) are both sensitive to glucose deprivation, in particular MIA PaCa-2, undergoing cell death, and, in different extend, are both protected by FSK treatment because of its ability to restore Complex I activity [15]. Moreover, both cell lines express an oncogenic K-ras that has been shown to promote cell metabolic rewiring and in particular glycolysis [24, 37]. As shown in Figures 6(a) and 6(b) Piericidin A treatment, used at concentration of 10 nM for 8 hours, did not affect survival of both cell lines grown in 25 mM glucose, as measured by Trypan Blue vital staining. On the contrary, as previously observed for

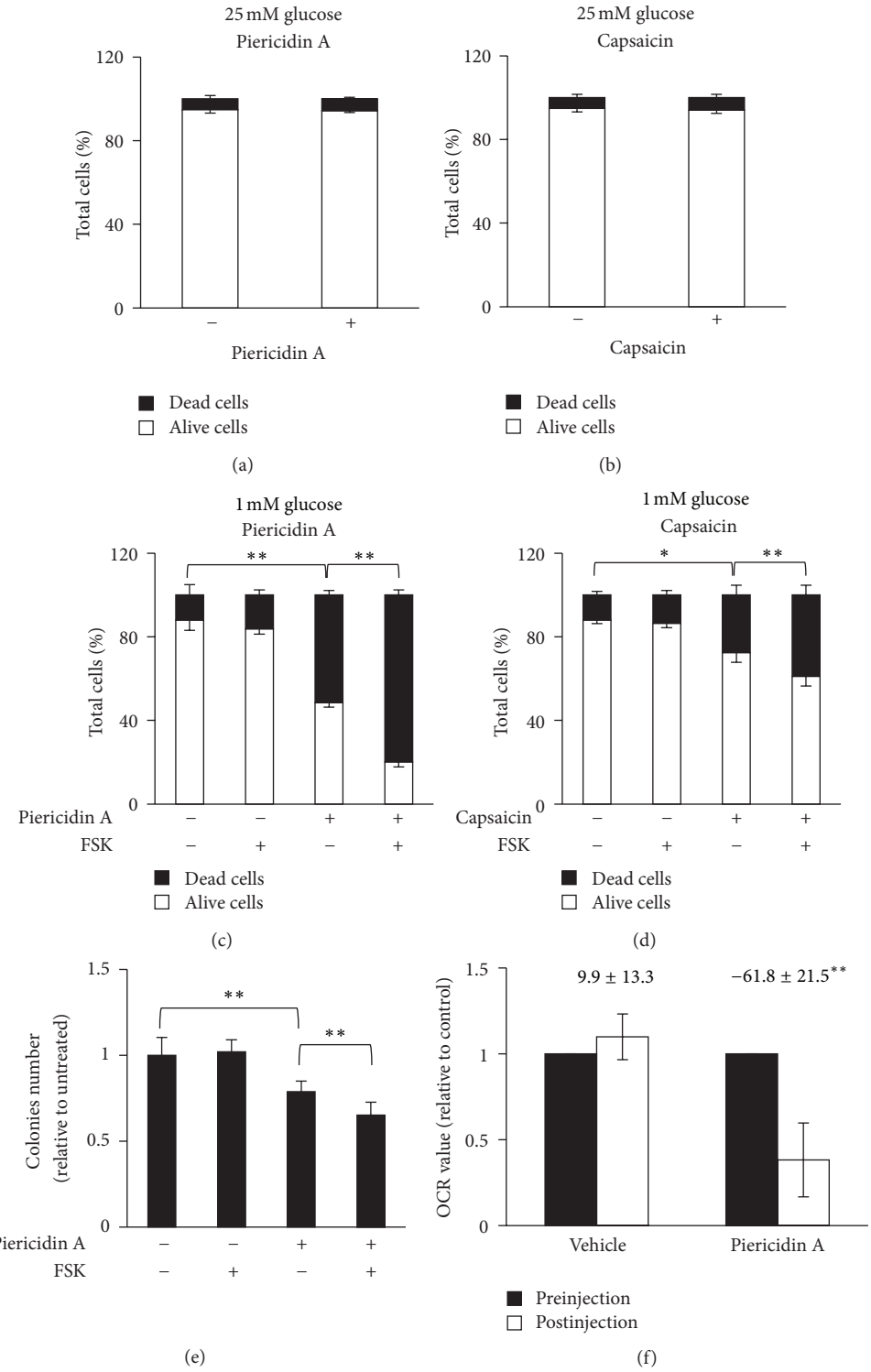

Figure 4: The mitochondrial Complex I inhibitors piericidin A and capsaicin induce cell death in MDA-MB-231 cells as observed with rotenone. Viable cell count using Trypan Blue was performed after treatment with 5 nM piericidin A or 100 μM capsaicin at 48 hours of culture in different growth conditions. (a)-(b) MDA-MB-231 cells were cultured in 25 mM glucose and counted after 2-hour treatment with piericidin A (a) or capsaicin (b). (c)-(d) MDA-MB-231 cells were cultured in 1 mM glucose and treated with (c) piericidin A or (d) capsaicin, 10 μM FSK, or FSK together with Complex I inhibitors. In the last case, cells were pretreated with FSK for 1 hour and then piericidin A or capsaicin were added for 2 hours (see Figure 2(c) as example). After treatment cell count was performed. (e) After treatment with piericidin A 3×10^3 cells were plated in normal growth medium for clonogenic assay and after ≥12 days colonies were stained and counted. (f) OCR of MDA-MB-231 cells cultured in low glucose was determined by Seahorse XF24 analyzer 30 minutes after injection of vehicle or 5 nM piericidin A; the percentage of OCR variation after injection is reported. All data represent the average of at least three independent experiments (±s.d.); $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's t-test).

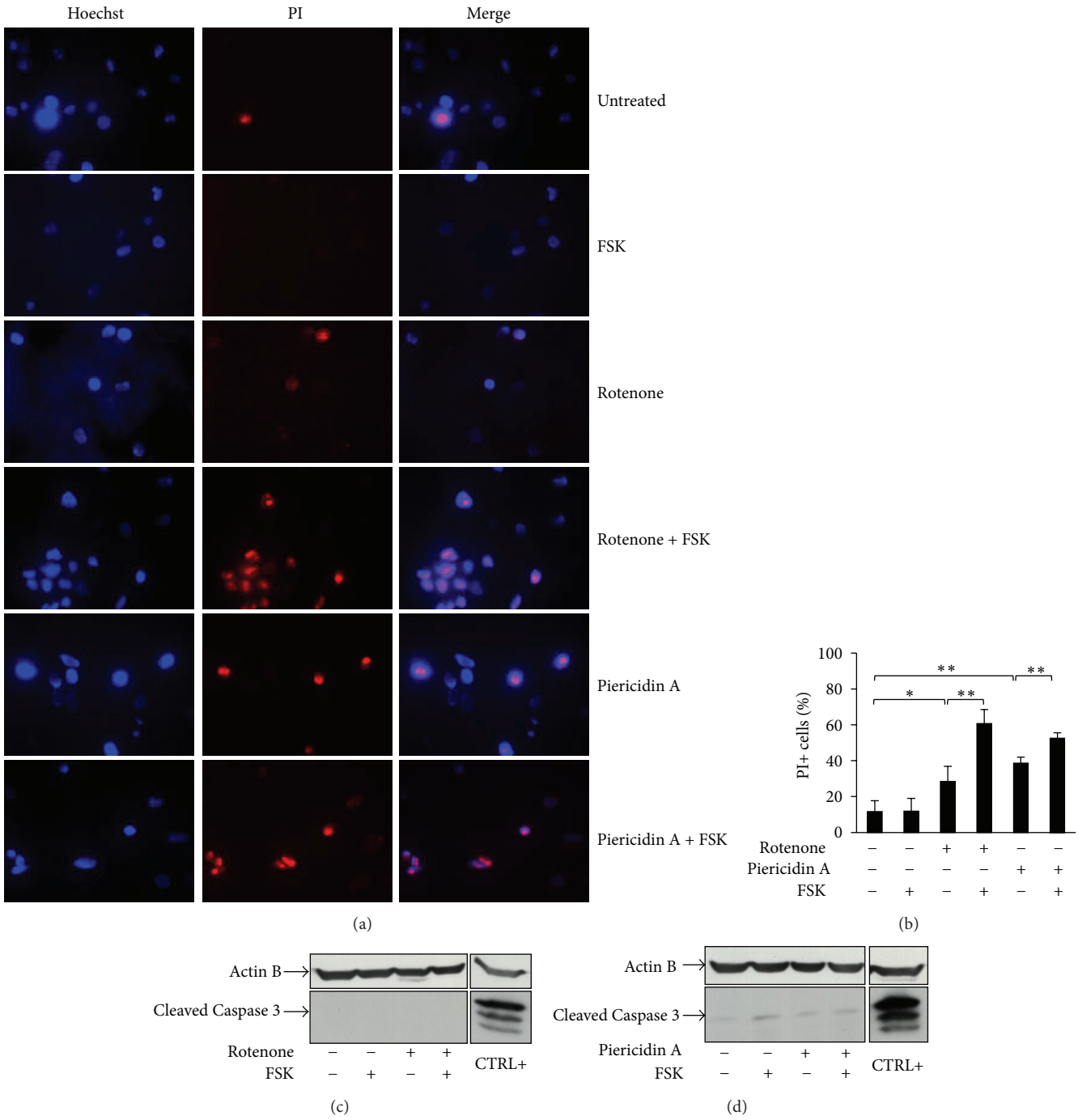

FIGURE 5: The combined treatment with Complex I inhibitors and FSK induces necrosis of MDA-MB-231 cells. Analysis of cell death was performed for MDA-MB-231 cells grown in low glucose and treated with 3 nM rotenone and 5 nM piericidin A alone or in combination with FSK. (a)-(b) Cells were stained with Hoechst and PI (a). As indication of necrosis cells that incorporated PI were counted and shown as percentage of the total cells (stained with Hoechst) considering at least 100 cells per sample (b). Data represent the average of at least three independent experiments (±s.d.); $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's t-test). (c)-(d) As indication of apoptosis the expression of the cleaved Caspase 3 was analyzed in cells treated with (c) rotenone and (d) piericidin A. As apoptotic control MDA-MB-231 cells were treated with thapsigargin (CTRL+). As loading control the expression of Actin B was evaluated. Data are representative of three independent experiments.

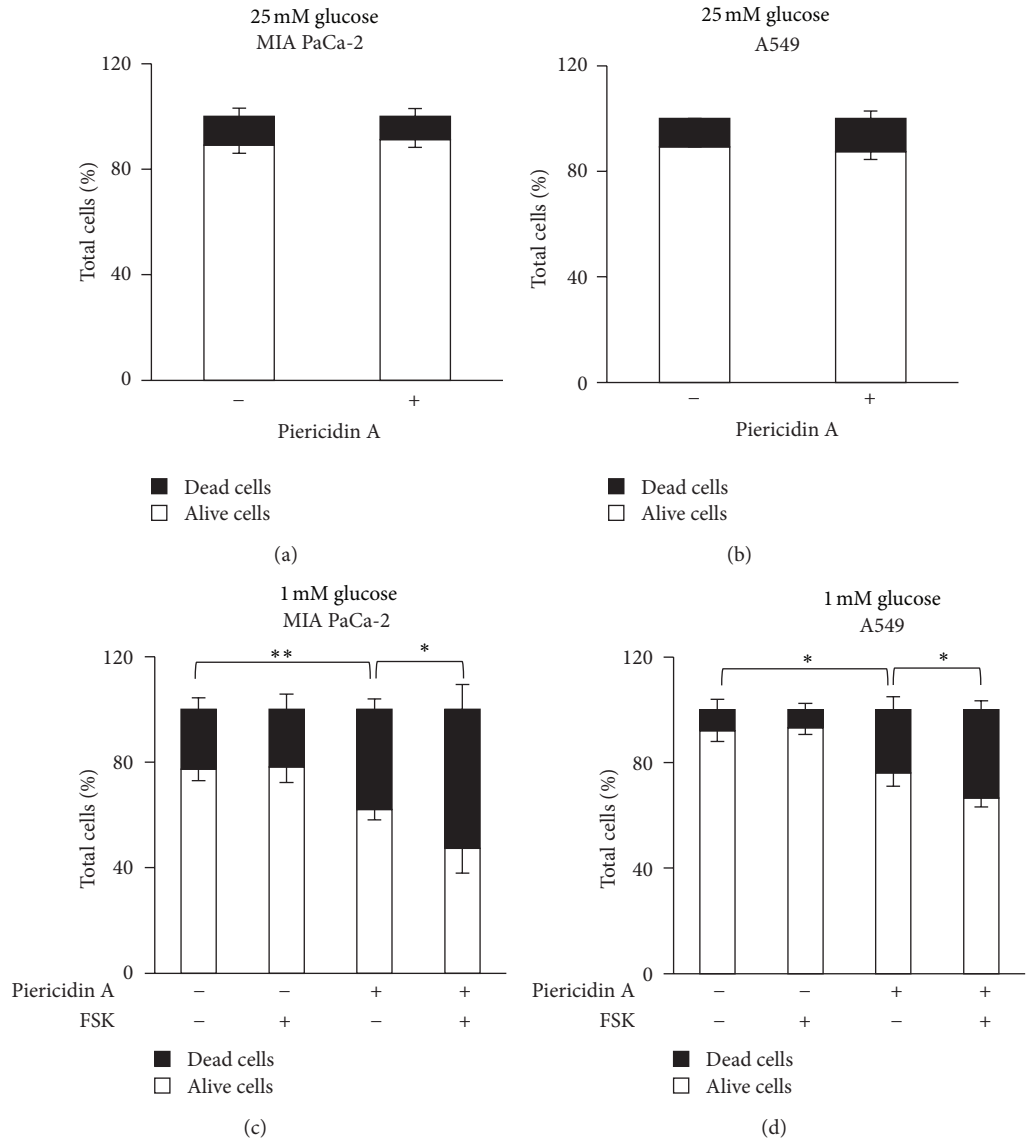

Figure 6: Piericidin A and low glucose synergize in inducing death also of pancreatic and lung cancer cells. Viable cell count using Trypan Blue was performed after treatment with 10 nM piericidin A in different growth conditions. (a)-(b) MIA PaCa-2 (pancreatic, A) and A549 (lung, B) cancer cells were cultured in 25 mM glucose and counted after 8-hour treatment. (c)-(d) MIA PaCa-2 (c) and A549 (d) cells were cultured in 1 mM glucose and treated with piericidin A, 10 μM FSK, or FSK plus piericidin A. For the cotreatment experiments, cells were pretreated with FSK for 1 hour and then piericidin A was added for 8 hours (see Figure 2(c), as example). After treatment cell count was performed. All data represent the average of at least three independent experiments (±s.d.); $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's t-test).

MDA-MB-231 cells, its addition in glucose depletion led in both cell lines to an increase of cell death that was stronger in more glycolytic cells MIA PaCa-2 (37.9%) (Figure 6(c)) than in A549 (23.9%) (Figure 6(d)). Notably, combined treatment with FSK further increased the percentage of cell death, reaching a value of 52.6% in MIA PaCa-2 (Figure 6(c)) and 33.4% in A549 (Figure 4(d)). Interestingly, in MIA PaCa-2 cells an increase of cell death was already observed in cells grown in low glucose as compared to high glucose (22.6% versus 10.8%), confirming their major dependence on glucose availability as compared to A549 cells.

3.7. Oligomycin-Dependent Cell Death of MDA-MB-231 Cells Increases in Glucose Deprivation. To further confirm the major sensitivity of cancer cells to inhibitors of mitochondrial function in a condition of low glucose, we decided to use also the mitochondrial ATP synthase inhibitor oligomycin. MDA-MB-231 cells are considered a cell line with a high glycolytic rate and a low level of respiration [15, 24]. For this reason we supposed that under treatment with oligomycin these cells could undergo a limited effect in high glucose and a significant effect in low glucose, since the latter condition is characterized by acute stimulation of respiration. MDA-MB-231 cells, cultured in 25 mM glucose, were treated for 1

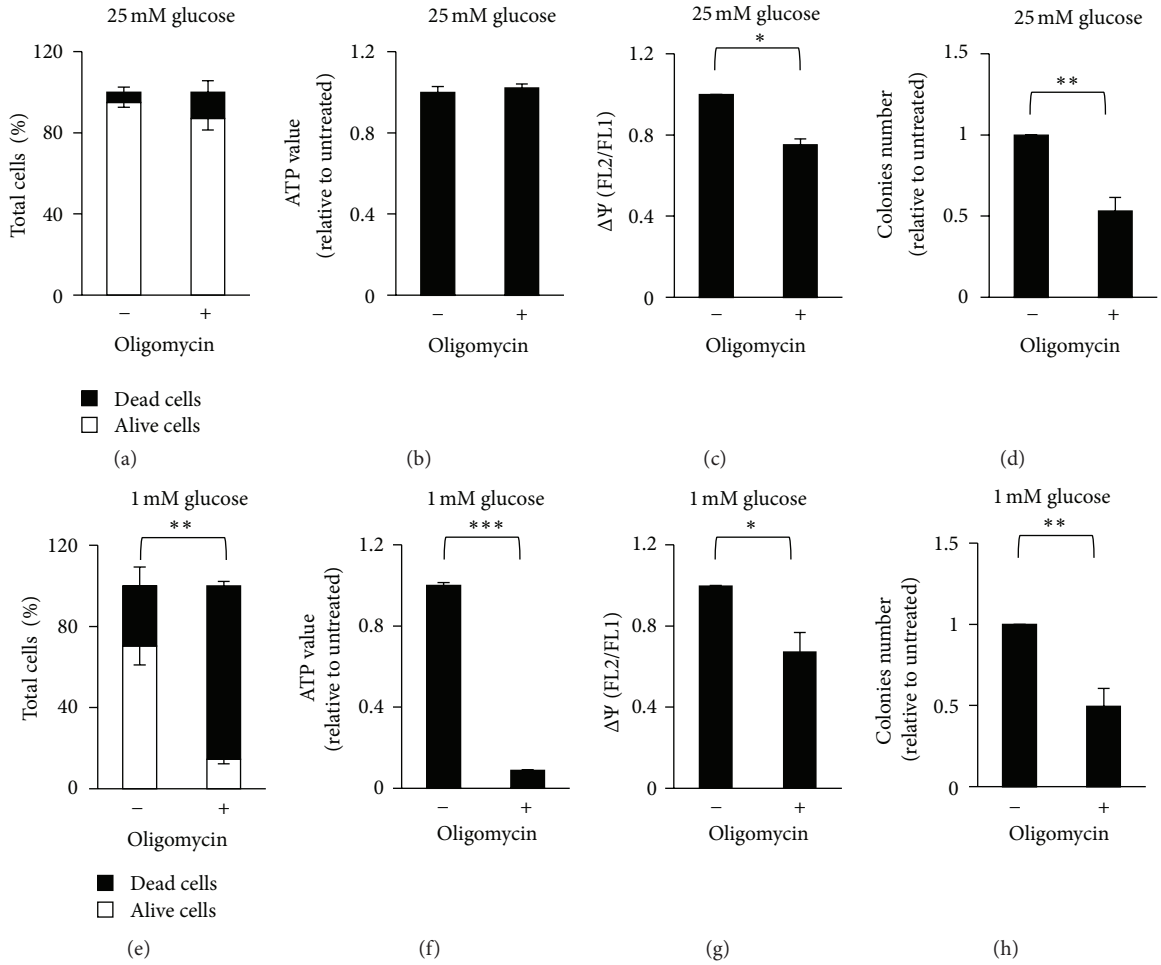

Figure 7: Oligomycin treatment induces a decrease of MDA-MB-231 cell viability especially in low glucose growth. MDA-MB-231 cells were cultured in (a)–(d) 25 mM and (e)–(h) 1 mM glucose and treated for 1 hour with 5 μM oligomycin at 48 hours of culture. After treatment different parameters were investigated in untreated (−) and treated (+) cells: viable cell count by (a), (e) Trypan Blue Staining, (b), (f) intracellular ATP levels, and (c), (g) mitochondrial potential. After treatment 3×10^3 cells were also plated in normal growth medium for clonogenic assay and after ≥12 days colonies were stained and counted (d), (h). All data represent the average of at least three independent experiments (±s.d.); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's t-test).

hour with 5 μM oligomycin and then analyzed. As shown in Figures 7(a) and 7(b) both cell viability and total intracellular ATP were not changed by the treatment, whilst a slight decrease of mitochondrial potential and a consistent decrease of colony formation ability were observed (Figures 7(c) and 7(d)). On the other hand, MDA-MB-231 cells grown in low glucose and treated with oligomycin showed a strong increase of cell death as indicated by Trypan Blue viable count (Figure 7(e)). Moreover, a complete depletion of intracellular ATP (Figure 7(f)), associated with a partial reduction of mitochondrial potential (Figure 7(g)), was observed. All these parameters were accompanied by a further decrease in the ability to form colonies as compared to cells grown in high glucose (Figure 7(h)). Taken together, these data indicate that in glucose shortage glycolytic cancer cells show a stronger and forced dependence on mitochondrial respiration that make them very sensitive to inhibitors of mitochondria function.

4. Discussion

Recently, different therapeutic approaches based on targeting tumor mitochondria have been proposed [8]. In fact, since this organelle is central both as producer of cellular ATP and as central regulator of apoptosis, it may be considered a good therapeutic hit [38]. The primary metabolic function of mitochondria is OXPHOS, an energy-generating process that couples the oxidation of respiratory substrates with ATP production. Besides ATP synthesis, mitochondria are involved in several other key metabolic processes such as oxidative decarboxylation of pyruvate, tricarboxylic acid cycle, and fatty acids oxidation. In addition, mitochondria take part in intracellular homeostasis of calcium and phosphate as well as in the balance of NAD^+/NADH. Besides their central role in metabolic activity, more recently mitochondria have been shown to have a central role also in the cascade of

events that leads to programmed cell death. In fact mitochondria represent a central checkpoint of this process by integrating various signals coming from endogenous factors (ions, metabolites, second messengers), from endogenous signaling proteins (kinases and phosphatases), and from exogenous factors (nutrients, oxygen). Therefore the strong interconnection between metabolism and apoptosis and the central role of mitochondria in both processes have led to an explosion of interest in connecting such pathways to the pathophysiology of cancer.

In this report, we have decided to utilize the main metabolic alterations of cancer cells, namely hyperglycolytic phenotype (Warburg effect) and mitochondria dysfunction, as targets for combined treatments aimed to specifically kill cancer cells. In fact, as shown by a number of groups, cancer cell proliferation and tumor aggressiveness correlate with an enhanced glycolysis and a low mitochondrial respiratory chain activity [3, 31], and positive or negative modulation of OXPHOS activity, depending on the metabolic state of cancer cells, appears to reduce tumor growth [39, 40]. Herein by using a glycolytic human breast cancer cell line, namely MDA-MB-231, grown in limiting glucose availability, and some natural inhibitors of mitochondria activity, we show that Complex I inhibition associated with an acute stimulation of respiration, due to glucose depletion, induces specifically necrotic cancer cell death (Figures 1, 3, and 5). Notably, this finding has been observed by using three different Complex I inhibitors [32] that strongly support our results (Figures 4 and 5). Moreover, we show that this cell death process, induced by the treatment with Complex I inhibitors, is activated also in other three cancer cell lines, mouse K-ras transformed fibroblasts, human MIA PaCa-2 pancreatic cancer cells, and human A549 lung adenocarcinoma cells. Importantly, such cell death mechanism is strongly dependent on glycolytic rate of the cancer cells. In fact MIA PaCa-2 cells, known to have a higher glycolysis as compared to A549, appear to be more sensitive to the treatment with inhibitors (Figure 6). Our results are interesting also because our and previously published data indicate that, at the used concentrations, rotenone (3 nM), capsaicin (100 μM), and piericidin A (5 nM) have no effect on immortalized fibroblasts (Figure 2), on normal pancreatic cells [41] and on dopamine neurons [28], respectively. This reduced or absent effect on normal cells is an important characteristic for exploiting these compounds for cancer therapy. Regardless of the mechanism of action of the three molecules, we show that the sensitivity to them increases upon glucose depletion that reflects the dependency of the cells on glycolysis. We suppose that less glycolytic cells, such as immortalized fibroblasts or normal cells, will be also less sensitive to these treatments. Since we observed a necrotic cell death (Figure 5), we attribute the synergistic effects more to ATP depletion than a rapid decrease of mitochondrial potential. However we cannot exclude an increase of ROS levels, as shown by other authors, as a consequence of Complex I inhibition [17, 25]. In fact, it is possible that stimulation of mitochondrial activity upon glucose depletion in the deranged respiratory system of malignant cells results in an increased production of oxidants, which may overwhelm cellular antioxidant protections and lead to cell death. Further experiments exploring this point will be addressed in the future.

From our studies, in particular from results obtained with FSK, an important point emerges: stimulation of mitochondrial activity and restoration of an ATP generating mechanism more similar to nonmalignant cells, might be an efficient tool in anticancer strategy. In particular, shifting cellular metabolism towards mitochondrial ATP production might overcome the positive effects on glycolytic pathway of oncogenes like K-ras, Akt and HIF-1 [42]. The idea is not completely new, since other authors have addressed this point by redirection of pyruvate towards oxidation in the mitochondria. In fact, inhibition of pyruvate dehydrogenase kinase (PDK) by DCA, or lactate dehydrogenase (LDH) by RNAi, has been shown to shift metabolism from glycolysis to glucose oxidation and to strongly reduce cancer cell viability and tumor growth [18, 43]. Our results with FSK suggest a similar mechanism in which reactivation of the mitochondrial function associated with glucose depletion and mitochondrial Complex I inhibition strongly affects cancer cell survival. Therefore, strategies involving the manipulation of both glycolytic and mitochondrial pathways might be useful to eradicate cancer cells. Other information in such a direction was derived also by experiments with oligomycin, an inhibitor of mitochondrial ATP synthase. We show that it is able to enhance cancer cell death in low glucose as compared to high glucose (Figure 7). In fact, we observed that in normal glucose conditions it does not induce a strong reduction of cell viability, although it is able to reduce the capability to form colonies. We can suppose that such a long-term effect is due to the inhibition of the reverse action of ATPase [44] that in fact is reflected in the mitochondrial potential decrease not accompanied with loss of ATP. On the other hand, the stronger effect of oligomycin on MDA-MB-231 cells in glucose deprivation is experimental evidence of their forced dependence on OXPHOS in such condition, exploitable by mitochondrial targeting therapies. Moreover, from another point of view, these data could suggest that inhibition of residual mitochondrial activity of cancer cells will further upregulate glycolysis and hence lead to their death by glucose depletion. This finding has been observed in lung carcinoma, in which oligomycin treatment upregulates glycolysis, increasing their dependence on this metabolic pathway [45].

Taken together our results provide a rationale for the use of mitochondrial inhibitors in cancer cells exploiting cancer cell fragility versus glucose depletion. In addition they point to an energetic switch from glycolysis to OXPHOS as an important therapeutic approach, since normal cells appear to be resistant to such combined treatments.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

New Players for Advanced Prostate Cancer and the Rationalisation of Insulin-Sensitising Medication

Jennifer H. Gunter,[1] Phoebe L. Sarkar,[1] Amy A. Lubik,[1,2] and Colleen C. Nelson[1,2]

[1] *Australian Prostate Cancer Research Centre-Queensland, Institute of Health and Biomedical Innovation, Queensland University of Technology, Translational Research Institute, Princess Alexandra Hospital, 199 Ipswich Road, Brisbane, QLD 4102, Australia*
[2] *Vancouver Prostate Centre, Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada V6H3Z6*

Correspondence should be addressed to Colleen C. Nelson; colleen.nelson@qut.edu.au

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Obesity and type 2 diabetes are recognised risk factors for the development of some cancers and, increasingly, predict more aggressive disease, treatment failure, and cancer-specific mortality. Many factors may contribute to this clinical observation. Hyperinsulinaemia, dyslipidaemia, hypoxia, ER stress, and inflammation associated with expanded adipose tissue are thought to be among the main culprits driving malignant growth and cancer advancement. This observation has led to the proposal of the potential utility of "old players" for the treatment of type 2 diabetes and metabolic syndrome as new cancer adjuvant therapeutics. Androgen-regulated pathways drive proliferation, differentiation, and survival of benign and malignant prostate tissue. Androgen deprivation therapy (ADT) exploits this dependence to systemically treat advanced prostate cancer resulting in anticancer response and improvement of cancer symptoms. However, the initial therapeutic response from ADT eventually progresses to castrate resistant prostate cancer (CRPC) which is currently incurable. ADT rapidly induces hyperinsulinaemia which is associated with more rapid treatment failure. We discuss current observations of cancer in the context of obesity, diabetes, and insulin-lowering medication. We provide an update on current treatments for advanced prostate cancer and discuss whether metabolic dysfunction, developed during ADT, provides a unique therapeutic window for rapid translation of insulin-sensitising medication as combination therapy with antiandrogen targeting agents for the management of advanced prostate cancer.

1. Obesity, Type 2 Diabetes and Prostate Cancer

1.1. Obesity and Cancer Risk. Worldwide rates of obesity have doubled in a generation with a global estimate of ~500 million obese adults (with an additional 1.5 *billion* overweight) being followed by a generation of 40 million overweight children [1]. In both industrialised and developing countries these staggering numbers pose a soaring economic and health care burden as a result of chronic comorbidities including increased rates of cardiovascular disease, hypertension, stroke, and type 2 diabetes (T2DM) [2].

Obesity is also a risk factor for a growing number of cancers. Retrospective observational studies and meta-analyses, using body mass index (BMI; mass (kg)/height (m^2)) as a measure of adiposity, have demonstrated an increased risk of breast, ovarian, colorectal, bladder, kidney, and endometrial cancers with increasing BMI [3–6]. Similarly, obesity attributes a 12% increased risk of prostate cancer diagnosis [3] although studies have varied, with some showing a weak association (relative risk (RR): 1.05 [7]), or a significant risk of high-grade and metastatic cancers, (RR: 1.22–1.55) but not low-grade prostate cancer [8]. These differences may be accounted for by differences in detection bias (using cohorts of closely monitored patients, for example, during diabetes screening), differences in time of "disease-free" follow-up and patient age [9]. Also, these studies may be limited by the use of BMI as a measurement of adiposity, underscored by the recent finding that specific measurement of visceral fat volume, the more metabolically compromised

fat depot in obesity, may provide a much stronger statistical correlate with disease-free survival in cancer patients than BMI [10]. Adding to these statistical differences, there are more practical difficulties of prostate cancer detection in obese subjects. Measurements of prostate specific antigen (PSA), a serum biomarker used for screening, which can also be elevated in nonmalignant disease such as benign prostatic hyperplasia (BPH) or with aging, can be difficult to interpret in obese patients; both lower levels of PSA, due to increased blood volume and increased PSA levels concomitant with increased incidence of BPH [11], have been reported in obese men [12]. Notwithstanding, a recent study by Fowke et al. [9] has found that the association between obesity and prostate cancer persists when these factors are taken into account.

1.2. Obesity and Cancer Progression. What is indisputable from the epidemiology is the impact of obesity on cancer behaviour. Obesity is consistently identified as a significant risk factor for more aggressive disease and an independent predictor of recurrence and cancer-specific mortality for breast [13], endometrial, ovarian [14], and bladder cancer [5] as well as prostate cancer [9, 15]. Men with higher BMI are more likely to be diagnosed with higher-grade cancers and higher Gleason scores and suffer an increased incidence of recurrence [3, 7, 15, 16] and increased cancer-specific mortality than men with a healthy BMI [16–18].

The molecular link(s) between obesity and malignancy is the subject of current research and has been recently reviewed [19–21]. Obese adipose tissue creates a hypoxic environment, as well as an overproduction of ROS resulting in oxidative and ER stress [22]. In addition, many bioactive molecules are altered in obesity which could contribute to neoplasia and cancer progression [21] including lipid mediators, inflammatory cytokines, and hormones/growth factors. As adipose tissue expands, a proinflammatory environment develops with increased secretion of cytokines such as IL-6, TNFα, and MCP1, from both adipocytes and resident immune cells. These, in turn, act as chemoattractants for further immune cells and thus create a feed-forward loop, perpetuating immune cell infiltration and cytokine production. Cell signalling is impacted, particularly insulin signalling in local and peripheral tissue (e.g., muscle and liver) leading to systemic insulin resistance [23] and sustained elevated circulating insulin levels.

Dysregulated lipid flux in obesity results in decreased HDL cholesterol and elevated LDL, circulating levels of triglycerides and free fatty acids which have been shown to promote prostate cancer cell survival [24, 25]. Inflammatory lipid mediators such as arachidonate and downstream signalling lipids, such as eicosanoids, prostanoids, and leukotrienes, are also increased [21, 26] and could potentially impact tumour cell biology; arachidonate can also promote steroid hormone production in prostate cancer cells [27].

Altered hormonal profiles are also a hallmark of metabolic dysfunction with modulated adipokine production and secretion, including increased leptin, proportional to adipose mass, while adiponectin shows an inverse relationship [28]. Elevated leptin levels have been associated with breast, prostate and colon cancer progression, and leptin promotes *in vitro* cell proliferation and invasion [9, 29], as well as independently modulating inflammation. Reduced adiponectin, which purportedly puts a brake on malignant cell proliferation, has also been observed in a range of cancers [30] including prostate cancer.

However, it is hyperinsulinaemia, as a result of insulin resistance in classical metabolic tissues, which has been identified as a highly significant risk factor to progression of prostate and other cancers [14]. In prostate cancer, elevated insulin or C-peptide levels (used as a normalised surrogate) have recently been shown to significantly correlate with high-grade prostate cancer and worse patient prognosis [16, 31–36], more significantly than BMI alone, suggesting that at least part of the effect of increased BMI on prostate cancer mortality is related to coincident hyperinsulinaemia [33]. Similarly, a strong association with high C peptide and the development of high-grade prostate cancer of Gleason grade 7 or greater has been identified, independent of BMI [36]. Baseline insulin levels at time of prostate cancer diagnosis have also been shown to be the most significant predictor of lethal prostate cancer, strongly suggesting that insulin is a major factor in prostate cancer progression associated with metabolic dysfunction [35]. Chronically elevated insulin facilitates in tumour tissues increased activation of mitogenic, anabolic, and prosurvival pathways with the increased levels of insulin and concomitant elevated insulin-like growth factor 1 (IGF-1) [37, 38]. We have also recently reported that insulin regulates the expression of novel gene transcripts/splice variants in tumour tissue [39]. In addition, many tumour types, including prostate tumours, upregulate the expression of related insulin receptor (INSR), IGF-1 receptor (IGF-1R), and hybrid INSR/IGF-1Rs [31, 40] further promoting insulin-driven cancer survival pathways [41]. And notably, an increased ratio of INSR/IGF1R expression has recently been described in prostate tumours and adjacent tissue [42], which suggests specifically that insulin signalling plays a key role in these tumours.

Together, the altered hormonal and inflammatory milieu of obesity may contribute significantly to cancer growth and progression via promoting mitogenesis (e.g., leptin, IGF-1, insulin), angiogenesis (e.g., VEGF, IL6, IL8), and invasion (e.g., leptin, IL6, PAI-1, CCL5, CCL2) [19].

1.3. Diabetes and Cancer Risk. Obesity is a major risk factor for the development of T2DM, a condition defined by hyperglycaemia in a background of insulin resistance in metabolic organs (e.g., muscle and liver). Diabetes generally develops as pancreatic β-cells are unable to respond adequately to increased insulin required to maintain normoglycaemia. Diabetes has been independently associated with increased risk of several cancers including colon, pancreatic, and breast cancer [43, 44]. In contrast, some studies suggest that diabetes may be protective of prostate cancer risk [45–47], perhaps due to the typically decreased levels of androgens in these men; prostate tumours are fuelled by androgens for growth and survival, and testosterone levels are typically lower in men with diabetes [46–48]. However, this is also confounded by a lack of separation of men taking diabetic control agents, such as metformin [46, 49]. Although diabetes is associated with

a lower incidence of prostate cancer, postdiagnosis, diabetes increases the risk of cancer-specific mortality [50], suggesting that similar risk factors contributing to cancer progression are involved in prostate cancer as other tumour types. Large cohort studies of diabetic patients are partly compromised by the fact that many studies reporting the relative risk of cancer with diabetic status have not separately defined the proportion of type 1 and type 2 diabetes within these studies, nor analysed the cohorts individually or based on medications [46, 49, 51]. While highlighting the association between hyperglycaemia *per se* and prostate cancer, metabolic risk factors associated with T2DM with potential links to cancer, such as hyperinsulinaemia and inflammation, are likely to lose statistical impact by combining diabetic subtypes; the rate of obesity in T2DM is 52% (86% overweight), compared to 16% in type I diabetes (55.3% overweight), and, as such, the metabolic contribution to malignancy, described above, would be expected to have significantly different impact [52]. Furthermore, type 1 diabetes is characterised by a *loss* of insulin and use of exogenous insulin analogues whereas T2DM is treated with a range of medications including metformin, sulphonylureas, and insulin which vary in cancer risk profiles [53–55], some conferring protection, (metformin) and others increasing risk (sulphonylureas and insulin analogues) [45, 53]. Indeed, this observation has led many to speculate on the potential utility of diabetes treatments as adjuvant cancer therapeutics.

2. Emergence of Insulin-Sensitising Drugs in Cancer

The observation of increased cancer risk with obesity and diabetes has led to the somewhat more hopeful speculation of potential therapeutic benefit of insulin-sensitizing drugs with the major clinical outcome of lowering systemic insulin levels. Studies have shown some cancer survival benefit in type 2 diabetes patients treated with the biguanide and metformin compared to those treated with insulin or sulphonylureas (stimulate insulin secretion from pancreatic β-cells) [53, 56–58]. In addition, the thiazolidinedione class of PPAR agonists [59], the lipase inhibitor, orlistat, and cholesterol-lowering statin class of drugs [60, 61] have also been shown to associate with lower cancer risk. A growing body of *in vitro* evidence is beginning to provide mechanistic detail to these observations.

2.1. Metformin. Metformin, which works in part by activating AMP-activated protein kinase (AMPK), is used clinically in obese and diabetic patients to normalise circulating insulin levels primarily via reduced hepatic glucose output and improve insulin/receptor interactions. Recent clinical studies have suggested that metformin may improve patient outcomes in prostate and other cancers [33, 53, 62–64]. Amongst diabetic populations, metformin has the lowest cancer mortality profile when compared to sulphonylurea or insulin treatment [53, 56–58, 63], in a dose and duration of treatment-dependent manner.

Apart from normalising systemic insulin, the intracellular effects of metformin in tumour cells have been the subject of recent study and review [41]. Metformin can potentiate several pathways to prevent tumour cell growth and invasion, primarily via blocking anabolic pathways such as lipognesis and protein translation, preventing mitosis and increasing sensitivity to chemotherapy.

The activation of AMPK in cancer cells blocks cell proliferation by negatively regulating mammalian target of rapamycin (mTOR) control of protein synthesis [43, 65–67]. Selective suppression of translation of mRNAs-encoding cell cycle regulators within the mTOR/EIF4E pathway by metformin has been recently described [68], and the expression of many genes involved in mitosis, such as tubulins, histones, and kinesins is decreased by metformin [69]. AMPK acts as an intracellular energy sensor, responding to fluctuations in the ratio of AMP and ATP. In times of energy deficit (high AMP:ATP ratio), the activation of AMPK blocks anabolic pathways and promotes the generation of ATP (e.g., oxidative phosphorylation, β-oxidation). Thus, metformin effectively blocks lipogenesis by inhibiting activation or expression of lipid biosynthesis enzymes such as acetyl Co-A carboxylase (ACC) and fatty acid synthase (FASN), key regulators of the metabolic reprogramming identified in prostate cancer [70]. Likewise, the enzyme responsible for steroid (estrogen) production, aromatase, is decreased in breast adipose tissue with metformin use [71].

Another proposed mechanism of metformin is preventing pathways which lead to invasion and metastasis evidenced by the demonstration of metformin to prevent invasiveness in cancer models [72, 73]. Similarly, it has been reported in pancreatic cancer cells that metformin reversed cell invasion by facilitating the reexpression of critical RNA species required for phenotypic maintenance [74]. Based on these results, targeting AMP-activated protein kinase (AMPK) has been proposed as a therapeutic strategy in cancer [75, 76].

2.2. Lipid Modulators: Orlistat and Statins. The pancreatic lipase inhibitor, orlistat, has its primary effect on reducing hydrolysis of triglycerides and preventing the absorption of dietary free fatty acids. Its major clinical benefit in obesity is preventing caloric intake, thus, reducing circulating lipid levels and improving insulin sensitivity [77]. FASN expression is upregulated in a number of cancers including ovarian, breast, and prostate [78–80] so the discovery that orlistat was a novel inhibitor of FASN [81] triggered numerous studies into the efficacy of orlistat as a therapeutic agent for cancer. orlistat has been shown to prevent *in vitro* and *in vivo* growth of the prostate cancer cell line PC3 and to induce apoptosis. [81]. The inhibition of FASN with orlistat also causes cell cycle arrest and induces caspase-mediated apoptosis [82] and has also been shown to increase sensitivity to chemotherapeutic compounds [83, 84].

Statins lower cholesterol levels by inhibiting a critical enzyme for cholesterol synthesis, HMGCoA reductase (HMGCR), which is upregulated in CaP and breast cancer tissue [85]. The role of HMGCR in cholesterol synthesis, and

a precursor to steroid hormone synthesis, is likely to be an important link in the association between statin use and a lower rate of prostate cancer recurrence [26, 86, 87]. Indeed, it has been shown that statin use inversely correlated with the risk and progression of prostate cancer [88, 89]. In a large-scale study by Loeb et al. men on statins were older and had high BMI at time of radical prostatectomy, but, despite these increased risk factors, the patients on statins had lower PSA, as well as lower Gleason score, lower tumour volumes, smaller surgical margins, and less risk of biological recurrence [88]. Other studies demonstrate a significant decrease in advanced and metastatic disease [88, 90].

2.3. Thiazolidinediones. Thiazolidinediones (TZDs) are a class of insulin-lowering (insulin-sensitising) drugs which activate the PPAR family of nuclear receptors, with predominant affinity for PPARγ, highly expressed in prostate cells [91]. Several TZDs are used clinically to treat type 2 diabetes, including pioglitazone, troglitazone, rosiglitazone, and ciglitazone. Their use in other diseases with hallmark hyperinsulinaemia (e.g., polycystic ovarian syndrome and some lipodystrophies) is the subject of an ongoing research [92]. A large meta-analysis comparing TZD use and cancer incidence [93] found a decreased risk of colorectal, lung, and breast cancers with TZDs, but specifically, the use of pioglitazone—which extended protection to prostate and renal cancer as well. This finding has also been demonstrated in other studies where TZD use is associated with improved survival of diabetic prostate cancer patients [59]. In prostate cancer cell models, troglitazone reduced *in vitro* and *in vivo* cell growth via ERK-dependent regulation of p21 and cMyc [94] and inducing apoptosis [80]. Prostate-specific knockdown of PPARγ resulted in the dysregulation of cell cycle control and lipid signalling networks [95]. In ovarian models, differential efficacy of ciglitazone and troglitazone (but not rosi- or pioglitazone) was observed with respect to cell cycle arrest, increased caspase activation, and evidence is now mounting that this may occur via PPARγ-independent mechanisms [96, 97]. In prostate cancer cells TZDs induced cell senescence, via PPARγ-dependent (rosiglitazone) and -independent (ciglitazone) mechanisms [98]. Studies in lung cancer cell models have reported TZD treatment works directly on cancer cells to decrease pathways that lead to invasion such as epithelial-to-mesenchymal transition (EMT) via Smad-dependent and -independent mechanisms [99]. Taken together, there is compelling evidence that current antidiabetes treatments offer therapeutic benefit in cancer.

2.4. Exogenous Insulin Analogues. The efficacy of insulin-lowering therapies in reducing risk and progression of cancer is highlighted by the converse, that is, increased risk of cancer in diabetic patients treated with insulin analogues. These include fast-acting analogues such as Aspart and Lispro with similar INSR but reduced IGF1R-binding affinities compared to native insulin [100], and Glargine and X10 with increased IGF1R affinity compared to insulin, and sustained binding kinetics [101, 102]. Nevertheless, the clear benefit of good glucose control in diabetes is worth bearing in mind in regard to cancer risk.

3. Prostate Cancer as a Candidate for Insulin-Lowering Therapy

3.1. Advanced Prostate Cancer. At current rates, prostate cancer will affect one man in seven and is the leading cause of cancer deaths in men in western countries [103]. For rapidly advancing, localised disease, patients are generally treated with radical prostatectomy or radiation therapy with a high rate of success (>90%) [104]. However, up to 25% of patients will recur, heralded by a biochemical recurrence measured as a rising PSA, and require secondary treatment. Prostate tumours depend on androgen signalling for growth and survival, and this dependence has rationalised the standard treatment for metastatic prostate cancer, androgen-deprivation therapy (ADT), for decades [105, 106]. The most common method of ADT uses luteinising hormone-releasing hormone (LHRH) agonists or antagonists to medically disrupt hypogonadal feedback loop to inhibit testicular testosterone production, or to a lesser degree direct orchiectomy. An initial surge of testosterone in response to LHRH agonists is often blunted with coadministration of androgen receptor (AR) antagonists (e.g., bicalutamide and flutamide). ADT results in anticancer response and prolongs cancer control; however, after a median time of 12 to 33 months, the cancer resurges, with a second PSA rise, despite castrate levels of serum testosterone (<20 ng/dL) [107]. This disease stage is referred to castrate-resistant prostate cancer (CRPC) and traditionally has had limited effective treatment options, finding that effective therapy for CRPC has been the focus of intense and productive research in recent years with a surge of new agents approved and, in development, many of which re-targeting in new ways the androgen axis.

4. Current Therapy for CRPC

While chemotherapies such as docetaxel [108, 109] and cabazitaxel [110] provide some survival benefit in metastatic CRPC, therapeutic strategies are increasingly focussing on the inhibition of androgen signalling as evidence mounts that CRPC continues to utilize androgens for tumour growth and driving cell survival pathways [111–113]. There are several mechanisms by which prostate tumours may reactivate androgen signalling, including gain-of-function mutations or alternative splicing of the androgen receptor (AR) that broaden its range of ligands to other steroid hormones (e.g., progesterone, estrogen, and cortisol), antiandrogens (e.g., flutamide and bicalutamide) [114–116], or confer ligand-independent activity [117]. The development of a hypersensitive receptor, via overexpression of AR and/or receptor stabilization, provides a second mechanism that results in AR activation even at low levels of androgens [118–121]. A further mechanism by which CRPC maintains AR signalling is by producing its own androgens from both adrenal conversion and intratumoural intracrine steroidogenic pathways. The observation that, despite low serum levels, intraprostatic

levels of DHT and testosterone were high in men treated with ADT [122], up to 50% of intraprostatic levels measured in eugonadal men [122–125], led to the finding by us and others that, under androgen deprived conditions, enzymes required for conversion of adrenal androgens and *de novo* intratumoural androgen synthesis are upregulated in prostate cancer cells [113, 126, 127]. Thus, no longer reliant on testicular androgens, AR signalling can resume within the tumour microenvironment, leading to the development of CRPC. These factors combined have rationalised the recent surge in new antiandrogens for the treatment of CRPC, which target the AR and inhibit activity, or block rate-limiting enzymes in *de novo* steroid synthesis.

4.1. New Inhibitors Targeting the AR. A tranche of new-generation antiandrogens have recently emerged from preliminary clinical trials with promising results; MDV3100 (Medivation Inc.), a small molecule AR antagonist, has recently been FDA approved for secondary hormone treatment for CRPC under the trade name of Enzalutamide. MDV3100 binding the AR blocks ligand binding, impairs nuclear translocation of the receptor, and induces a conformational change that prevents AR transcription and cofactor recruitment [128]. MDV3100 has from 5- to 10-fold greater binding affinity for the AR than bicalutamide or Casodex, but, unlike bicalutamide, has no partial agonist activity [128] and, excitingly, may also block constitutively active AR splice variants [129]. A second AR-antagonist, ARN509 (Aragon Pharmaceuticals), similarly binds AR, prevents nuclear translocation and transcription, and, in early clinical trials, has shown promise for efficacy at lower doses than MDV3100 [130].

4.2. New Inhibitors Targeting Steroid Synthesis. Antiandrogen therapies are also aimed at inhibiting androgen biosynthesis. Androgens are synthesized from cholesterol via steroidogenesis. Multifunctional enzyme CYP17A1 catalyzes two important steps in the steroidogenesis pathway: the conversion of progestogens to androgen precursors and subsequent conversion to androgens (DHEA and androstenedione). DHEA and androstenedione are then converted to testosterone via 17-β-hydroxysteroid dehydrogenase (17BHSD) and then to DHT via RDH5. Currently, both ketoconazole (a pan-CYP inhibitor, Figure 1) and aminoglutethimide (inhibitor of CYP11A1) are used to block key enzymes in the androgen synthesis pathway in combination with ADT.

Improved, specific CYP17A1 inhibitors such as abiraterone (Janssen) FDA, TAK-700 (Takeda/Millenium Pharmaceuticals), and TOK-001 (Tokai Pharmaceuticals) are now in various stages of clinical assessment for adjuvant use with LHRH agonists. abiraterone, unlike previous CYP17 inhibitors, has a 3-pyridyl substitute and 16,17 double bond, which makes it a highly specific, potent, and irreversible inhibitor of both the hydroxylase and lyase activity of CYP17A1 [131–133]. abiraterone acetate, the oral drug precursor of abiraterone, has been FDA approved with trade named Zytiga for CRPC treatment, in settings of postdocetaxel [134] and now prechemotherapy [135]. TAK-700 is a nonsteroidal imidazole, a potent inhibitor of CYP17A1 lyase activity, with weak inhibition of hydroxylase activity and therefore may not require concomitant control of rise in mineralocorticoids as is needed for abiraterone. TAK-700 is currently undergoing Phase I/II clinical trials. A third molecule, 17-benzoimidazole TOK-001, has combined CYP17/AR inhibitor activity and has shown promising results in preclinical studies including a remarkable decrease in AR protein expression and regression of *in vivo* xenograft tumours [136].

While much progress has been made in the emergence of new drugs suppressing AR activity in prostate cancer, the unfortunate reality is that, even in these early trials, patients have become resistant to these new therapies [137]. Both abiraterone and MDV3100, currently the only new-generation drugs with FDA approval, show survival benefits of 4.6 months [134] and 4.8 months [138], respectively, in the postdocetaxel setting. In the prechemotherapy setting, abiraterone treatment offers an increased time to biochemical recurrence of 11.1 months [134, 135]. Early trial results show that MDV3100 has improved efficacy, with an extra 5.3 months to biochemical failure compared to postdocetaxel [138]. The persistent capability of CRPC to become resistant to various means of suppression of AR signalling suggests that a multipronged approach to cancer treatment is required and leads to the research question: what drives prostate cancer aggressiveness and CRPC progression?

5. Metabolic Dysfunction Caused by ADT Accelerates CRPC

Metabolic dysfunction is a well-established side effect of ADT [34, 139]. The response to suppression of testosterone in men, irrespective of patient BMI at treatment commencement, includes gain of fat and loss of muscle mass, increased LDL and triglycerides, hypertension, and increased fasting glucose [140]—metabolic symptoms which significantly overlap the comorbidities of obesity. Abrupt withdrawal of androgens by ADT results in hyperinsulinaemia and loss of insulin sensitivity reflected by increased homeostatic model assessment (HOMA) score, within 2 weeks [141], suggesting a direct effect of ADT and is independent of fat mass and age [139, 142]. Major findings from recent studies [36, 143] and our own recent pilot study of men receiving ADT demonstrated a strong trend between elevated C-peptide and more rapid progression to CRPC. This phenomenon appears to be due to a direct inverse relationship that exists between the testosterone and insulin hormonal axes in men [144–149]; testosterone is inversely linked to insulin sensitivity and insulin-sensitising medication increases testosterone [59, 150]; however, the exact mechanisms of crosstalk between these pathways are not well understood [151, 152].

Traditionally, insulin has been considered a hormone-controlling metabolic regulation; however, the pathways activated by insulin, including phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/MAPK pathways, have many well-characterised downstream effects relevant to CRPC progression, including the inhibition of apoptosis (e.g., via FOXO and BAD-mediated pathways) [118, 153] and stimulation of cell proliferation (e.g., via mTOR) (Figure 1, *insulin-signalling pathways*) [40, 154]. Hyperinsulinaemia, secondary to ADT

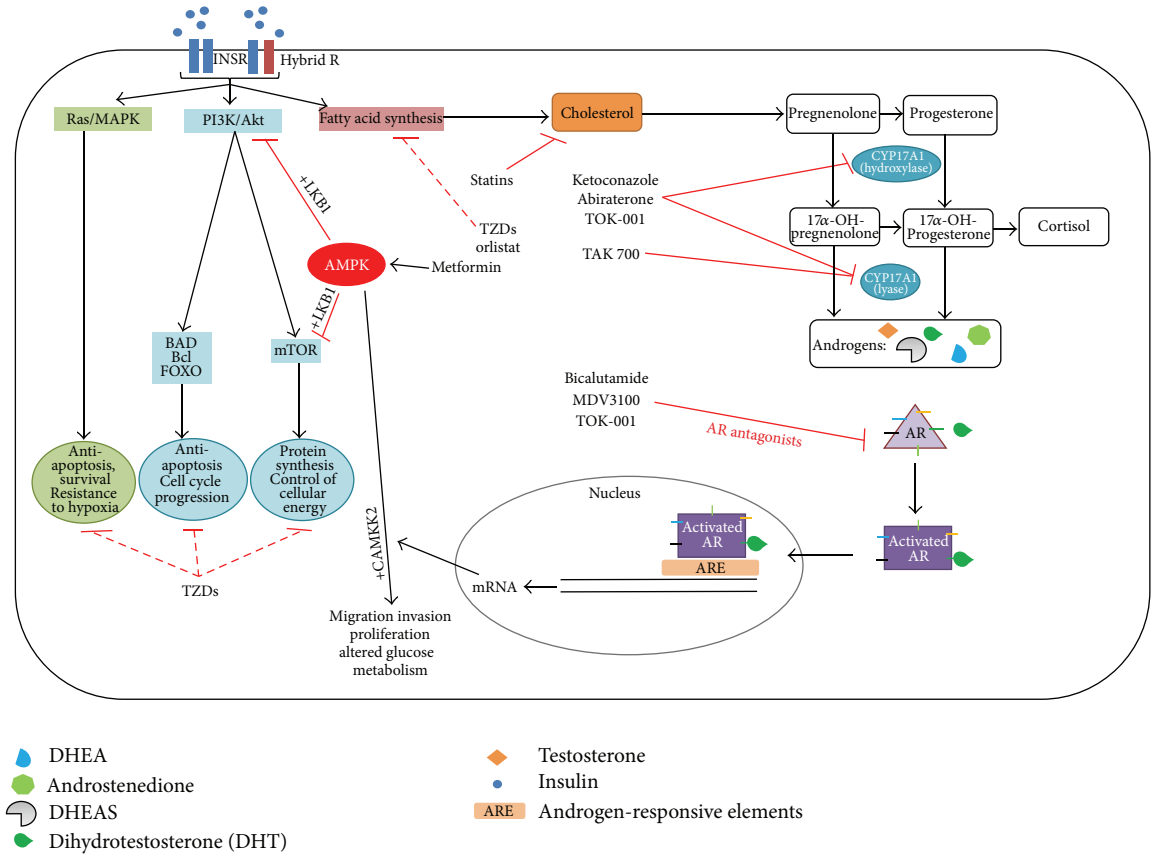

Figure 1: Therapeutic regimens combining insulin-sensitizing drugs and androgen-signalling inhibitors may be beneficial for treating prostate cancer progression following ADT. Androgen signalling is targeted using direct AR inhibitors. Bicalutamide (Casodex) and MDV3100 directly bind AR to prevent activity. Ketoconazole, abiraterone (Zitega), and TOK-001 have dual inhibition of CYP17A1 lyase/hydroxylase activity. In addition TOK-001 directly blocks AR activity. TAK-700 inhibits CYP17A1 lyase activity only and may not require concomitant control of rise in mineralocorticoids as is needed for abiraterone. Hyperinsulinaemia would be expected to increase insulin signalling in prostate cancer cells. Insulin can accelerate *de novo* androgen synthesis [153], which provides the AR with several suitable ligands (Key) and allows AR-mediated transcription of genes needed for CRPC progression. Additionally, insulin signalling may activate several AR-independent pathways (e.g., antiapoptosis and cell proliferation). Insulin-sensitizing drugs, such as metformin, orlistat, thiazolidinediones, and statins effectively block insulin-induced effects such as proliferation, lipid, and cholesterol synthesis and, hence, may be effective for the treatment of prostate cancer. The role of AR on AMPK-mediated cell response is still unclear and may be dependent on availability of cofactors (LKB1 versus CAMKK2).

treatment, would be expected to increase the activation of these pathways in the relatively insulin-sensitive prostate tumour. Furthermore, increased insulin signalling would be facilitated by increased INSR expression in advanced prostate cancer [31] (Lubik, Gunter, Nelson, unpublished data). We have recently demonstrated that insulin accelerates intratumoural androgen synthesis [155], a major pathway contributing to progression to CRPC. But, insulin is expected to upregulate a number of pathways leading to ADT failure [156, 157]. Regulation of molecules by signalling downstream of insulin/IGF-1R has also been implicated in CRPC progression, including COX2 [158–160], and nuclear AR chaperone, Hsp27 [161, 162]. Notably, obesity and diabetes may independently regulate these molecules [163–165]. Moreover, serum from obese mice has been shown to induce an invasive phenotype in prostate cancer cell lines [166] suggesting that metabolic changes associated with obesity (including elevated insulin) may drive metastatic transformation. However, chronic insulin signalling in the context of ADT might be complex and highly differentiated from those observed in other cancers [167]. Potential crosstalk between AR and insulin-signalling cascades [168–171] emphasises the potential insulin-driven CRPC progression. And recent studies have implicated the PI3K/Akt pathway activation to inhibit AR signalling [172] resulting in androgen-independent growth. Several other growth factors and cytokines upregulated in obesity, such as IL-6, IL-8, IGF-1, and TGFβ, may also influence prostate cancer cell proliferation [173–175].

6. A Future for Combined Therapy

Epidemiological evidence, coupled with the upregulation of insulin-signalling components and insulin-mediated upregulation of CRPC pathways in prostate cancer cells, has collectively rationalised a multidisciplinary approach to treating

advanced prostate cancer that incorporates antiandrogen therapy with simultaneous treatment of metabolic side effects induced by ADT.

Metformin has been the first drug to be explored in this area, with ongoing clinical trials using metformin during active surveillance (NCT01733836) and in combination with ADT (NCT01620593) testing the efficacy of metformin in stabilising/normalising circulating insulin levels. Metformin has a well-characterised safety profile and, while it effectively decreases hyperglycaemia, does not affect blood glucose levels in nondiabetic patients. Metformin may also act directly on tumour cells to reduce cancer cell proliferation via the inhibition of anabolic pathways such as lipogenesis starving the major bioenergetic pathway in prostate cancer cells. However, the ability of the major intracellular target of metformin, AMPK, to potentiate insulin action on cell growth and survival may have more complex regulation in prostate cancer cells via interaction with AR-regulated genes; activated AMPK may potentiate increased prostate cancer cell proliferation and migration when activated downstream of the androgen receptor (AR) [176, 177] under the control of a master regulator calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) [177]. AR directly regulates CAMKK2 and is highly expressed in normal prostate with elevated expression in both AR-sensitive and CRPC models of prostate cancer [176, 177]. In studies where metformin activation of AMPK results in cessation of cancer cell growth, signalling is through LKB-1 tumour suppressor [178] suggesting that AMPK is a potentially bifunctional modulator, particularly relevant in the prostate cell, dominated by AR-regulated transcriptional landscape, and conversely may be dysregulated with AR as occurs in prostate cancer. The ability of insulin to accelerate *de novo* steroid synthesis in prostate cancer cells suggests that drugs targeting steroidogenesis in combination with insulin-lowering therapies would be beneficial; trials combining abiraterone and metformin are yet to commence (NCT01677897).

Atorvastatin (statin) is also currently under examination for treatment in prostate cancer in combination with ADT (NCT01555632). Statins target cholesterol synthesis, the substrate for steroidogenesis and in adrenocortical cells and the ovaries, have been shown to decrease steroidogenic enzymes CYP11A1, HSD3B, and CYP17A1 [179]. Statins were able to slow or halt progression to castrate resistance in LNCaP xenograft models [180] and inhibited prostate cancer growth with greater potency in androgen-deprived condition. The clinical benefit of metformin combined with statin for treatment of biochemical recurrence following primary treatment failure (PSA increase following prostatectomy or radiation therapy) is also under clinical investigation (NCT01561482), targeting lipid/cholesterol metabolism and hyperinsulinaemia in an effort to slow progression.

The dual activity of orlistat, reducing dietary lipid absorption and as an inhibitor of FASN, could also have an adjunct role to play in mitigating the side effects of ADT which may promote cancer progression. orlistat could potentially be used to reduce systemic dyslipidaemia, normalising free fatty acid and cholesterol levels and thereby reducing substrate for tumour metabolism and biosynthesis. However, dietary fats make up only a fraction of the lipid available to prostate cancer cells, which almost universally upregulate FASN [181].

7. Conclusion

Prostate cancer is the most common cancer in men [103] and, given its long natural history and with onset beginning in 5-6 decades of life, will continue to rise and plague our ageing population. At the same time we face the growing epidemic of obesity while the risk of aggressive prostate cancer is increased 3-fold with obesity. First-line therapies for localised cancer will fail in nearly 25% of prostate cancer patients, and these men will subsequently be treated with ADT [182]. While effectively treating prostate cancer, ADT induces hyperinsulinaemia [182–185] which independently acts on prostate cancer to upregulate lipid and steroid synthesis and contribute to CRPC progression and which may promote metastases, tumour growth, and treatment resistance. Standard chemotherapeutic agents have had limited benefit in CRPC, and there has been quite promising development of next-generation therapies targeting androgen synthesis and directly AR in combination with ADT. These new targeted agents are generally well-tolerated and significantly improve survival; however, most patients ultimately fail, highlighting the urgent need to understand mechanisms underlying treatment resistance and find rationally informed combined and/or sequential treatment options. An existing toolbox of well-tolerated insulin-lowering therapies has accumulated over several decades to treat type 2 diabetes and metabolic complications associated with obesity. Currently, ADT-induced hyperinsulinaemia is not addressed in prostate cancer patients, despite a significantly increased risk of cardiovascular and cancer-related mortality in these patients [186]; the combination of ongoing research and clinical trials will determine the benefit of adjunct antiinsulin therapy to current standard and emerging prostate cancer treatments.

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Review Article

Functions of BCL-X_L at the Interface between Cell Death and Metabolism

Judith Michels,[1,2,3] Oliver Kepp,[1,3] Laura Senovilla,[1,3] Delphine Lissa,[1,3] Maria Castedo,[1,2,3] Guido Kroemer,[1,4,5,6,7] and Lorenzo Galluzzi[3,4,7]

[1] *INSERM, U848, Institut Gustave Roussy, Pavillon de Recherche 1, 39 Rue Camille Desmoulins, 94805 Villejuif, France*
[2] *Université Paris Sud/Paris XI, 94805 Villejuif, France*
[3] *Institut Gustave Roussy, 94805 Villejuif, France*
[4] *Université Paris Descartes/Paris V, Sorbonne Paris Cité, 75006 Paris, France*
[5] *Metabolomics Platform, Institut Gustave Roussy, 94805 Villejuif, France*
[6] *Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP, 75015 Paris, France*
[7] *Equipe 11 Labelisée par la Ligue Nationale Contre le Cancer, Centre de Recherche des Cordeliers, 75006 Paris, France*

Correspondence should be addressed to Guido Kroemer; kroemer@orange.fr and Lorenzo Galluzzi; deadoc@vodafone.it

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The BCL-2 homolog BCL-X_L, one of the two protein products of *BCL2L1*, has originally been characterized for its prominent prosurvival functions. Similar to BCL-2, BCL-X_L binds to its multidomain proapoptotic counterparts BAX and BAK, hence preventing the formation of lethal pores in the mitochondrial outer membrane, as well as to multiple BH3-only proteins, thus interrupting apical proapoptotic signals. In addition, BCL-X_L has been suggested to exert cytoprotective functions by sequestering a cytosolic pool of the pro-apoptotic transcription factor p53 and by binding to the voltage-dependent anion channel 1 (VDAC1), thereby inhibiting the so-called mitochondrial permeability transition (MPT). Thus, BCL-X_L appears to play a prominent role in the regulation of multiple distinct types of cell death, including apoptosis and regulated necrosis. More recently, great attention has been given to the cell death-unrelated functions of BCL-2-like proteins. In particular, BCL-X_L has been shown to modulate a number of pathophysiological processes, including—but not limited to—mitochondrial ATP synthesis, protein acetylation, autophagy and mitosis. In this short review article, we will discuss the functions of BCL-X_L at the interface between cell death and metabolism.

1. Introduction

According to current models, cell death most often proceeds via either of two relatively independent subroutines, apoptosis, and necrosis [1, 2]. For a long time, apoptotic and necrotic instances of cell death have exclusively been identified based on morphological criteria [2]. In addition, while apoptosis was believed to constitute the sole regulated (i.e., genetically encoded, and hence susceptible to pharmacological modulation) modality of cell death, necrosis was viewed as a purely accidental process [3]. Recently, a functional classification of cell death mechanisms, based on measurable biochemical features, has been proposed [1], and the concept of regulated necrosis has gained large consensus [4]. In this scenario, the true relevance of additional processes that were previously catalogued as *bona fide* cell death subroutines is being reevaluated. In particular, while macroautophagy (hereafter referred to as autophagy) turned out to constitute a prominent homeostatic and cytoprotective mechanism [5, 6], autophagic cell death (a lethal subroutine that is mediated, rather than merely accompanied, by autophagy) has been shown to occur in a limited number of, mostly developmental, scenarios [1, 7]. Along similar lines, mitotic catastrophe, a signaling cascade elicited in mitosis-incompetent cells that was initially viewed as a particular case of apoptosis [8], has recently been proposed to constitute an oncosuppressive mechanism with multiple functional outcomes, including cell senescence as well as apoptotic and necrotic cell death [9].

Apoptotic stimuli can be propagated via two distinct, but not entirely disjointed, molecular cascades: extrinsic

apoptosis, transducing lethal signals that originate in the extracellular microenvironment, and intrinsic (also known as mitochondrial) apoptosis, responding to perturbations of intracellular homeostasis [10]. Extrinsic apoptosis can be initiated either by the ligand-induced activation of plasma membrane death receptors (e.g., FAS/CD95, tumor necrosis factor receptor 1 (TNFR1)) or by so-called dependence receptors (e.g., deleted in colorectal carcinoma (DCC)), when the concentration of their ligands falls below a specific threshold [1]. Death receptors promote the activation of caspases (a class of cysteine proteases that play a central role in multiple instances of apoptosis) [11] via the formation of a multiprotein complex that includes—among other components—receptor-interacting protein kinase 1 (RIPK1), FAS-associated protein with death domain (FADD), cellular inhibitor of apoptosis proteins (cIAPs), and multiple isoforms of cellular FLICE-inhibitory protein (c-FLIP). Such a death-inducing signaling complex (DISC) allows for the proximity-induced autoactivation of caspase-8, in turn catalyzing the proteolytic maturation of caspase-3, the central effector of most cases of apoptosis [10]. The mechanisms whereby dependence receptors are connected to the execution of apoptosis have only recently begun to emerge and appear to involve caspase-9, a caspase that was long believed to exclusively regulate mitochondrial apoptosis [12].

Intrinsic apoptosis can be triggered by a plethora of perturbations in intracellular homeostasis, including—among others—DNA damage, oxidative stress, and cytosolic Ca^{2+} overload [10]. Independent of the initiating stimulus, the signaling cascades that mediate intrinsic apoptosis as well as the prosurvival signals that are generated alongside (to facilitate the reestablishment of homeostasis) are opposed to each other at the level of mitochondria [13–15]. If lethal signals prevail, the majority of mitochondria become permeabilized, an event that *de facto* seals the cell fate. Indeed, upon mitochondrial outer membrane permeabilization (MOMP), (i) the mitochondrial transmembrane potential ($\Delta\psi_m$), that is, the electrochemical gradient driving ATP synthesis as well as many other mitochondrial functions, is rapidly dissipated; and (ii) cytotoxic proteins that are normally secluded within the mitochondrial intermembrane space (e.g., cytochrome c; apoptosis-inducing factor (AIF), endonuclease G (ENDOG)) are released into the cytosol, where they promote the activation of caspases as well as of caspase-independent cell death executioner mechanisms. The former relies on the cytochrome *c*-elicited, dATP-, and apoptotic peptidase activating factor 1 (APAF1)-dependent assembly of the so-called apoptosome, a molecular platform for the activation of the caspase-9 → caspase-3 cascade. The latter involves the caspase-independent endonuclease activity of AIF and ENDOG as well as the bioenergetic and redox crisis that ensues $\Delta\psi_m$ dissipation [13, 14]. Of note, extrinsic and intrinsic apoptosis are not entirely disjointed. Indeed, while in some cell types (e.g., lymphocytes) the caspase-8 → caspase-3 cascade is sufficient to mediate death receptor-dependent apoptosis, in others (e.g., hepatocytes), this process requires the caspase-8-mediated cleavage of the BH3-only protein BID, generating a mitochondrion-permeabilizing fragment (see below) [16, 17].

Given its position at the frontier between cell life and death, it is not surprising that MOMP constitutes a highly regulated phenomenon. So far, two models have been put forward to explain MOMP in molecular terms [14, 18]. On one hand, MOMP has been suggested to originate at the mitochondrial outer membrane (OM), thanks to the pore-forming activity of multidomain proapoptotic members of the BCL-2 protein family, namely, BAX and BAK [14, 19]. On the other hand, it has been proposed that—in response to specific triggers—MOMP would stem from the so-called mitochondrial permeability transition (MPT), an abrupt increase in the permeability to small solutes of the mitochondrial inner membrane (IM). In this latter scenario, a critical role has been ascribed to the permeability transition pore complex (PTPC), a large molecular entity assembled at the junctions between the OM and the IM by several proteins, including (though presumably not limited to) voltage-dependent anion channels (VDACs), adenine nucleotide translocase (ANTs), and cyclophilin D (CYPD) [13, 18].

Importantly, antiapoptotic multidomain members of the BCL-2 protein family, including BCL-2 itself, BCL-X_L, and MCL-1, not only counteract the pore-forming activity of BAX and BAK by engaging in direct inhibitory interactions, but also (i) intercept upstream proapoptotic signals such as those mediated by BH3 only proteins like BAD, BID, BIM, and BBCR3 (best known as p53-upregulated modulator of apoptosis (PUMA)) [20, 21], (ii) bind to, hence regulating, several components of the PTPC, including VDAC1 and ANT [22–24], and (iii) prevent the generation of proapoptotic cytosolic Ca^{2+} waves, either by interacting with inositol 1,4,5-trisphosphate (IP3)-gated Ca^{2+} channels on the endoplasmic reticulum (ER) or by limiting the capacity of ER Ca^{2+} stores [25–27]. In addition, both pro- and antiapoptotic BCL-2-like proteins have recently been shown to modulate multiple processes that are not directly connected to the execution of cell death, including—among others—bioenergetic metabolism, mitochondrial functions, mitosis, and autophagy [28, 29].

Here, we discuss the multifaceted role of BCL-X_L, a prototypic antiapoptotic member of the BCL-2 family, at the hub between cell death and metabolism.

2. BCL-X_L and Cell Death

In humans, BCL-X_L is encoded by *BCL2L1*, a *BCL2*-related gene mapping to chromosome 20q11.21 [30]. *BCL2L1* was shown from the beginning to code for two distinct protein products, owing to the alternative splicing of *BCL2L1* mRNA: a cytoprotective factor of 233 residues (BCL-X_L) and a smaller polypeptide (170 residues) that exerts BCL-X_L-antagonizing functions (BCL-X_S) [30]. Similar to BCL-2, BCL-X_L contains four distinct BCL-2 homology (BH) domains (BH1–BH4) as well as a transmembrane region, through which it localizes—at least in part—to several membranous compartments, including the OM, the ER, and the nuclear envelope [30–32] The same does not apply to BCL-X_S, which lacks both the BH1 and BH2 domains [30, 33]. Of note, in most physiological settings the BCL-X_L-coding mRNA is expressed to higher levels than its BCL-X_S-coding counterpart [34]. Conversely, BCL-X_S often predominates in

situations of developmental and pharmacological cell death [35, 36]. Importantly, a caspase-generated cleavage product of BCL-X_L (lacking an N-terminal fragment) has recently been shown to mediate neuronal cell death in rodent models of ischemic brain injury [37], suggesting that chemical inhibitors of BCL-2-like proteins might also be employed (at least in selected circumstances) as cytoprotective agents.

BCL-2 and BCL-X_L were soon recognized as critical antiapoptotic factors, although this function was initially attributed to their ability to mediate antioxidant effects [38, 39]. This notion has quickly been abandoned in favor of the so-called "rheostat" model, proposing that BCL-2 and BCL-X_L would physically sequester their proapoptotic counterparts BAX and BAK in inhibitory interactions [40–42]. In the following decade, along with the discovery of several other members of the BCL-2 protein family, this model has been progressively refined to include the concepts of "activating" and "derepressing" BH3-only proteins [21]. According to current viewpoints, the former would promote MOMP by engaging in direct activatory liaisons with BAX and BAK, while the latter would do so by competitively displacing BAX and BAK from inhibitory interactions with BCL-2, BCL-X_L, and MCL-1 [21]. Of note, the core concept of the rheostat model as first theorized by Stanley Korsmeyer in 1993 [43], that is, that cell death is governed by the balance between pro- and antiapoptotic BCL-2 family members, has remained remarkably unmodified since its original formulation.

Nevertheless, during the last two decades, antiapoptotic BCL-2 family members, including BCL-X_L, have been shown to exert cytoprotective functions via a myriad of mechanisms that do not necessarily rely on their capacity to block the pore-forming activity of BAX and BAK although—at least in some instances—they do involve a BAX-/BAK-antagonizing effect. Similar to BCL-2, BCL-X_L prevents the generation of proapoptotic cytosolic Ca^{2+} waves by reducing capacity of ER Ca^{2+} stores, an effect that is antagonized by BAX and BAK [25–27]. Moreover, BCL-X_L has been shown to critically regulate the opening status of VDAC1, and hence of the PTPC, thus influencing MPT-dependent apoptotic cell death [23, 24, 44].

Of note, while some authors proposed that the MPT would stem from an unselectively open conformation of the PTPC [23], others concluded that the MPT would originate from the closed state of the pore [24, 44]. Irrespective of this controversy, which has not yet been fully resolved, recent data have confirmed a critical role for the interaction between VDAC1 and BCL-X_L in the antiapoptotic properties of the latter [45]. Interestingly, many other BCL-2 family members such as BAX, BAK, BID, and BCL-2 appear to interact with (and hence modulate the activity of) PTPC components (i.e., VDAC1, VDAC2, and ANT) [22, 23, 46, 47], suggesting that the crosstalk between these two systems might constitute a particularly important point of functional regulation. However, the actual relevance of the PTPC for the cellular demise in physiological settings remains matter of debate. Indeed, mice lacking one or more of the most critical PTPC components (including all VDAC and ANT isoforms known thus far) [48–50], with the single exception of *Ppif*$^{-/-}$ animals (lacking CYPD) [51–53], fail to exhibit remarkable cell death defects in response to ischemic, traumatic, and pharmacological challenges. Hence, it seems that—at least in physiological settings—the complex crosstalk between BCL-2 family members and the PTPC mainly modulates cell-death unrelated cellular functions and impacts on the cellular demise only via indirect circuitries (see below).

One of the most central regulators of apoptosis as triggered by perturbations of intracellular homeostasis such as DNA damaging conditions, imbalances in redox homeostasis and oncogenic stress is p53 [54]. Besides operating as a stress-responsive transcription factor that regulates the synthesis of both pro- and antiapoptotic proteins, including a large panel of BCL-2 family members (i.e., BAX, BAK, BAD, BID, PUMA, BCL-2, and BCL-X_L) [55], p53 can also exert apoptotic functions in a transcription-independent fashion [56, 57]. In particular, p53 has been shown to operate similar to BH3-only proteins, that is, to promote MOMP either by engaging in activatory (though labile) interactions with BAX [58] or by displacing BAX and BAK from inhibitory liaisons with BCL-2 and BCL-X_L [59]. In this setting, the mitochondrial pools of BCL-2 and BCL-X_L constitute the main target for the "derepressor" activity of p53 [59]. In addition, a cytoplasmic pool of BCL-X_L appears to work as a PUMA-sensitive inhibitor of p53, *de facto* operating at the interface between p53 transcriptional and transcription-independent functions [60]. Thus, BCL-X_L exerts cytoprotective effects not only as it antagonizes its proapoptotic counterparts but also as it counteracts the activity of p53. In addition, BCL-X_L has recently been reported to interact with the mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5) [61], a central effector of regulated necrosis [62, 63]. Hence, although Niture et al. did not address this question in a direct fashion, BCL-X_L may soon be discovered to regulate necrotic instances of cell death.

3. BCL-X_L and Metabolism

The hypothesis that BCL-2 family members, notably BAX, BAD, BCL-2, and BCL-X_L, would influence bioenergetic and intermediate metabolism began to gain consensus along with the discoveries that (i) these proteins interact with components of the PTPCs that, in physiological circumstances, regulate various facets of mitochondrial functions (e.g., ANT, VDAC, and glucokinase) [22–24, 44, 64], (ii) these proteins modulate Ca^{2+} homeostasis at the ER [25–27], and (iii) p53 not only operates as a potent proapoptotic factor in response to stress but also exerts an homeostatic control over metabolism [54]. In particular, BCL-X_L (i) reportedly preserves the physiological conformation of VDAC, hence promoting the exchange of metabolites, including ADP, across the OM [44]; (ii) functionally antagonizes BAD [42], which has been found to exert prominent metabolic functions by regulating a mitochondrial multiprotein complex that involves (among other enzymes) glucokinase, protein kinase A, and protein phosphatase 1 [64]; (iii) has been shown to lower the concentrations of Ca^{2+} ions within the ER, hence quenching the bioenergetic burst that normally results from

the opening of IP_3-gated Ca^{2+} channels [25–27]; and (iv) binds to cytoplasmic p53 in steady-state conditions [60], thus—at least theoretically—modulating its functions related to bioenergetic and redox metabolism [54, 65, 66].

BCL-X_L has also been demonstrated to regulate distinct facets of intermediate metabolism in a direct fashion. In neurons, a pool of BCL-X_L localized to the IM appears to physically interact with the β subunit of the F_1F_O ATP synthase, hence increasing its enzymatic efficiency, stabilizing the $\Delta\psi_m$ and—consequently—maximizing mitochondrial ATP generation [67, 68]. Similar functions have been attributed to a truncated variant of MCL-1 that localizes to the mitochondrial matrix [69]. In addition, the transfection-enforced overexpression of BCL-2 has been associated with increased oxygen consumption and higher rates of mitochondrial respiration [70, 71]. Taken together, these observations suggest a conserved role for BCL-2 proteins in the regulation of ATP synthesis. Of note, antiapoptotic members of the BCL-2 family have been suggested to exert prooxidant functions, at least under selected circumstances [70, 71]. Such a (slight) prooxidant state, presumably reflecting the ability of BCL-2 and BCL-X_L to stimulate mitochondrial respiration [67, 68, 70], appears to be linked to the interaction of BCL-2-like proteins with small GTPases of the RAC family [72, 73] and to exert cytoprotective effects by contributing to the maintenance of baseline energetic homeostasis [71].

Recent data have indicated that BCL-X_L operates (in a BAX- and BAK-independent manner) to limit the intracellular levels of acetyl-CoA [74]. Acetyl-CoA is not only a critical intermediate of the Krebs cycle, but also required for protein acetylation, including N-α-acetylation, that is, the posttranslational modification that consists in the addition of an acetyl moiety (provided by acetyl-CoA) to the N-terminus of nascent polypeptides [74]. Hence, high expression levels of BCL-X_L exert cytoprotective effects along with the establishment of a state characterized by decreased levels of virtually all the metabolites involved in the Krebs cycle (but not of glycolytic substrates) as well as by reduced extents of N-α-acetylation [74]. Although Yi and colleagues ascribed such an antiapoptotic state (which could be reversed by the exogenous supply of citrate and acetate) only to the inhibition of N-α-acetylation [74], reduced levels of reactive oxygen species (ROS), which constitute a normal byproduct of mitochondrial respiration, may equally well underpin (at least part of) the cytoprotective effects that originate from BCL-X_L metabolic functions [75]. In support to this notion, (i) the overactivation of several metabolic circuitries (including glycogenolysis and glutaminolysis) and the overgeneration of ROS have been linked to both apoptotic and necrotic cell death [13, 76]; and (ii) a predominantly glycolytic metabolism, as observed in cancer cells even in the presence of normal oxygen levels (i.e., the so-called Warburg effect), reportedly exerts cytoprotective effects as it increases the amounts of reduced glutathione (a potent antioxidant) [75]. Of note, the pyruvate kinase M2, a glycolytic enzyme variant that is known to sustain the Warburg effect [77], has been shown to stimulate the expression of BCL-X_L at the transcriptional level [78]. Although the cytoprotective transcription factor NF-κB may play a role in this setting [78], the precise molecular mechanisms underlying this phenomenon remain to be identified. In addition, protein acetylation has recently been involved in the regulation of autophagy (see below) [79–82], suggesting that BCL-X_L might exert a broad control over multiple cellular functions.

4. BCL-X_L and Autophagy

Autophagy is a catabolic pathway driving the lysosomal degradation of cellular constituents such as portions of the cytoplasm, protein aggregates, and dysfunctional/supernumerary organelles [83]. Under physiological conditions, autophagy plays a prominent role in the maintenance of intracellular homeostasis [5, 84, 85]. In addition, the autophagic flux is dramatically upregulated in response to a large panel of stress conditions, including (but not limited to) glucose and amino acid deprivation, hypoxia, intracellular pathogens, and cytotoxic xenobiotics [83, 86]. Although in some, mostly developmental, scenarios an autophagic program *de facto* mediates cell death [1, 7], stress-elicited autophagy near to invariably exerts prominent cytoprotective functions [87]. In line with this notion, both pharmacological and genetic maneuvers that block autophagy most often exacerbate, rather than limit, cell death as triggered by several distinct stimuli [88].

The stress-elicited upregulation of autophagy is a tightly regulated phenomenon, involving distinct molecular sensors and signal transduction cascades that impinge at various levels on the autophagic machinery. A detailed description of the proteins and factors that are involved in the regulation and execution of autophagy largely exceeds the scope of this paper and can be found elsewhere [83, 84, 87, 89]. Nevertheless, it is important to note that—in most (but not all) settings—autophagy critically relies on a class III phosphoinositide-3-kinase (PI3K) enzymatic activity [90]. In human cells, this function is mediated by phosphatidylinositol 3-kinase, catalytic subunit type 3 (PIK3C3, best known as hVPS34), which operates under the control of a multiprotein complex involving—among other interactors—the haploinsufficient oncosuppressor Beclin 1 [91, 92]. Importantly, by virtue of a *bona fide* BH3 domain [93], Beclin 1 can physically interact with antiapoptotic members of the BCL-2 protein family, including BCL-2 itself and BCL-X_L [94–96].

By interacting with Beclin 1, BCL-2 and BCL-X_L *de facto* prevent the stress-induced activation of autophagy [94]. In line with this notion, both "derepressor" BH3-only proteins (e.g., BAD) and chemical inhibitors of BCL-2-like proteins (e.g., ABT-737) have been shown to activate autophagy as they displace Beclin 1 from inhibitory liaisons with BCL-2 and BCL-X_L [94, 97]. Interestingly, some BH3-only proteins like BNIP3 have been shown to be critical for the execution of specific autophagic programs, such as the selective removal of damaged mitochondria (mitophagy) [5, 98], while their relevance in cell death regulation seems rather limited [99]. The binding of Beclin 1 to antiapoptotic BCL-2 family members can also be resolved by the phosphorylation of either binding partner [100–102]. Conversely, it seems that BCL-2 and BCL-X_L do not affect the steady-state levels of the autophagic flux in a direct fashion [100]. Of note, Tian et al. have recently

suggested that BCL-2/BCL-X_L-targeting compounds might activate a Beclin 1- and PIK3C3-independent autophagic program leading to cell death [103]. However, the authors failed to provide robust data to mechanistically explain their findings.

Besides a direct autophagy-modulatory function stemming from its interactions with Beclin 1 and other BCL-2-like proteins [94, 96], BCL-X_L is expected to regulate autophagy via less direct metabolic circuitries, notably as it (i) controls the efficiency of mitochondrial ATP production [67, 68], (ii) influences the exchange of critical bioenergetic metabolites (e.g., ATP and ADP) by PTPC components [24, 44], (iii) reduces the intracellular levels of acetyl-CoA [74], and (iv) interacts with the cytoplasmic pool of p53 [60, 65, 66]. Hence, the expression levels of BCL-X_L might also influence the autophagic flux in steady-state, as opposed to adaptive, conditions. This aspect of the crosstalk between BCL-X_L and autophagy warrants further investigation.

5. BCL-X_L and Other Cellular Functions

The implication of BCL-2 family members, including BCL-X_L, in cell death-unrelated processes is not limited to the aspects of cell biology discussed above [28, 29]. For instance, it has recently been shown that BCL-X_L is phosphorylated at multiple serine residues (including S49 and S62) in a cell cycle-dependent fashion [104, 105]. The mitotic kinase Polo-like kinase 3 (PLK3) appears to be responsible for the cell cycle-dependent phosphorylation of BCL-X_L at S49 (starting at the S phase and abruptly falling at the onset of mitosis) [104], whereas PLK1 and mitogen-activated protein kinase 9 (MAPK9) have been suggested to catalyze BCL-X_L phosphorylation at S62 in response to DNA-damaging agents, hence stabilizing a cell cycle arrest at the G_2 checkpoint [105]. Hence, similar to BCL-2 [106, 107], BCL-X_L plays a role in both physiological cell cycle progression and DNA damage-induced cell cycle checkpoints [104, 105].

A few years ago, rather unspecific inhibitors of BCL-2-like proteins such as ABT-737 and ABT-263 have generated an intense wave of enthusiasm and quickly entered clinical trials as part of antineoplastic regimens for the treatment of—mostly hematological—malignancies [108]. One of the most prominent on-target side effects of ABT-737 and ABT-263 turned out to be a dose-limiting thrombocytopenia [109, 110], linked to the fact that BCL-X_L is critical for the survival of platelets [111]. More recently, BCL-X_L has also been involved in the adhesive function of platelets [112, 113]. Hence, ABT-737 and ABT-263 appear to impair aggregation not only as they trigger the demise of a consistent fraction of circulating platelets but also as they exert consistent thrombocytopathic effects among residual platelets [113].

The implication of the BCL-2 protein family in mitochondrial dynamics as well as the actual relevance of mitochondrial fission/fusion events in apoptosis have been and still are the subject of a vivid debate [114–116]. While a detailed discussion of this topic largely exceeds the scope of this paper, it is worth noting that BCL- X_L has recently been shown to interact with (and stimulate the GTPase activity of) dynamin-related protein 1 (DRP1), a central component of the mitochondrial fission machinery [117]. In doing so, BCL-X_L appears to alter the mitochondrial function of neurons in a manner that stimulates the formation of synapses [117]. As DRP1 also participates in the execution of regulated necrosis [63], mitochondrial dynamics may constitute yet another point of control of cell death-related and -unrelated processes by BCL-X_L.

BCL-2 and BCL-X_L have been reported to negatively regulate the NLRP1 inflammasome, a supramolecular platform that is required for the full-blown activation of caspase-1—and hence the production of interleukin (IL)-1β and IL-18—in response to proinflammatory stimuli [118, 119]. In particular, the flexible loop domain of both BCL-2 and BCL-X_L (which is located between the 1st and 2nd α helices of the proteins) appears to engage in physical interactions with NLRP1, thereby blocking the capacity of the latter to bind ATP and oligomerize [118, 120]. Besides playing a critical role in innate immunity, inflammasomes are crucial for the translation of immunogenic cell death (a functionally peculiar form of apoptosis) into a robust adaptive immune response [121]. It is therefore tempting to speculate, yet remains to be formally proved, that the interaction between antiapoptotic BCL-2 family members and inflammasomes may constitute a promising therapeutic target for enhancing the immunogenicity of (cancer) cell death [121].

6. Concluding Remarks

Following an initial wave of interest on the role of BCL-2-like proteins in the regulation of apoptosis, several laboratories have refocused their attention on distinct aspects of the biology of pro- and antiapoptotic members of the BCL-2 family [28, 29]. During the last decade, this intense investigational effort has led to the identification of several processes that are modulated by BCL-2 family proteins independent of (or at least not directly impacting on) their cell death-regulatory functions [28, 29]. As discussed in this paper, BCL-X_L has been shown to exert a consistent degree of control on various aspects of bioenergetic metabolism, including mitochondrial ATP production, Ca^{2+} fluxes, autophagy, and protein acetylation, as well as on several other cellular and organismal processes such as mitosis, platelet aggregation, and synaptic efficiency (Table 1). Hence, similar to other BCL-2 family members, BCL-X_L appears to operate at critical hubs to coordinately control multiple cellular functions including the three-step switch between homeostatic metabolisms, adaptive responses to stress, and cell death [122]. In this scenario, it is tempting to speculate, yet remains to be formally demonstrated, that BCL-2-like proteins may have originated as regulators of non-apoptotic functions and only later in evolution may have acquired the capacity of control cell death. Irrespective of this unresolved issue, we expect that—similar to what happened (and is still happening) for p53 [54]—the number of cellular functions that are regulated by BCL-2 family members, including BCL-X_L, will grow further.

Abbreviations

AIF: Apoptosis-inducing factor

Table 1: Functions of BCL-X_L at the interface between cell death regulation and other aspects of the cell biology.

Interactor	Main localization	Notes(s)	Reference
BAD	Cytosol mitochondria	By antagonizing BAD, BCL-X_L modulates the metabolic functions of a mitochondrial multiprotein complex involving glucokinase, PKA, and PP1	[64]
Beclin 1	Cytosol Golgi network	BCL-X_L binds to Beclin 1, thus inhibiting stress-induced, but not baseline, autophagy	[94, 97, 100]
DRP1	Mitochondria	BCL-X_L interacts with DRP1, altering the mitochondrial function of neurons to stimulate the formation of synapses	[117]
F_1F_O ATP synthase	Mitochondria	BCL-X_L increases the enzymatic activity of the F_1F_O ATP synthase, hence, stabilizing the $\Delta\psi_m$ and maximizing mitochondrial ATP synthesis	[67, 68]
IP_3R	ER	BCL-X_L reduces Ca^{2+} concentration in the ER	[25–27]
Krebs's cycle	Mitochondria	BCL-X_L overexpression reduces the levels of virtually all TCA (but not glycolytic) intermediates, modulating both N-α-acetylation and autophagy	[74, 79–82]
MAPK9 PLK1	Cytosol nucleus	BCL-X_L is phosphorylated at S49 by PLK1 and MAPK9 in a cell cycle-dependent fashion	[104]
NLRP1 inflammasome	Cytosol	BCL-X_L inhibits the NLRP1 inflammasome, interfering with the secretion of IL-1β and IL-18	[118, 120]
p53	Cytosol mitochondria	BCL-X_L binds to p53, hence, inhibiting both its pro-apoptotic and metabolic functions	[54, 60, 66]
PKM2	Cytosol mitochondria	PKM2 stimulates the expression of BCL-X_L at the transcriptional level	[78]
PLK3	Cytosol nucleus	PLK3 phosphorylates BCL-X_L at S62 in response to DNA-damaging agents, favoring a cell cycle arrest at the G_2 checkpoint	[105]
RAC2	Cytosol plasma membrane	In some settings, antiapoptotic BCL-2 family members exert prooxidant functions, perhaps linked to their interaction with RAC2	[70–72]
VDAC1	Mitochondria	BCL-X_L promotes the exchange of metabolites between the cytosol and the mitochondrial matrix	[24, 44]

$\Delta\psi_m$: mitochondrial transmembrane potential; DRP: dynamin-related protein 1; ER: endoplasmic reticulum; IL: interleukin; IP_3R: inositol 1,4,5-triphosphate receptor; MAPK9: mitogen-activated protein kinase 9; PKA: protein kinase A; PKM2: pyruvate kinase M2; PLK: polo-like kinase 1; PP1: protein phosphatase 1; VDAC1: voltage-dependent anion channel 1.

ANT: Adenine nucleotide translocase
APAF1: Apoptotic peptidase-activating factor 1
BH: BCL-2 homology
cFLIP: Cellular FLICE-inhibitory protein
cIAP: Cellular inhibitor of apoptosis protein
CYPD: Cyclophilin D
DCC: Deleted in colorectal carcinoma
DISC: Death-inducing signaling complex
DRP1: Dynamin-related protein 1
ENDOG: Endonuclease G
ER: Endoplasmic reticulum
FADD: FAS-associated protein with death domain
IL: Interleukin
IM: Mitochondrial inner membrane
IP_3: Inositol 1,4,5-trisphosphate
MAPK9: Mitogen-activated protein kinase 9
MOMP: Mitochondrial outer membrane permeabilization
MPT: Mitochondrial permeability transition
OM: Mitochondrial outer membrane
PGAM5: Phosphoglycerate mutase family member 5
PI3K: Phosphoinositide-3-kinase
PI3KC3: Phosphatidylinositol 3-kinase, catalytic subunit type 3
PLK3: Polo-like kinase 3
PTPC: Permeability transition pore complex
PUMA: p53-upregulated modulator of apoptosis
RIPK1: Receptor-interacting protein kinase 1
ROS: Reactive oxygen species
TNFR1: Tumor necrosis factor receptor 1
VDAC: Voltage-dependent anion channel
$\Delta\psi_m$: Mitochondrial transmembrane potential.

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Review Article

PKM2, a Central Point of Regulation in Cancer Metabolism

Nicholas Wong,[1,2,3,4] Jason De Melo,[1,2,3,4] and Damu Tang[1,2,3,4]

[1] *Division of Nephrology, Department of Medicine, McMaster University, Hamilton, ON, Canada L8S 4L8*
[2] *Division of Urology, Department of Surgery, McMaster University, Hamilton, ON, Canada L8S 4L8*
[3] *Father Sean O'Sullivan Research Centre, St. Joseph's Healthcare Hamilton, Hamilton, ON, Canada L8N 4A6*
[4] *The Hamilton Center for Kidney Research, St. Joseph's Healthcare Hamilton, Hamilton, ON, Canada L8N 4A6*

Correspondence should be addressed to Damu Tang; damut@mcmaster.ca

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Aerobic glycolysis is the dominant metabolic pathway utilized by cancer cells, owing to its ability to divert glucose metabolites from ATP production towards the synthesis of cellular building blocks (nucleotides, amino acids, and lipids) to meet the demands of proliferation. The M2 isoform of pyruvate kinase (PKM2) catalyzes the final and also a rate-limiting reaction in the glycolytic pathway. In the PK family, PKM2 is subjected to a complex regulation by both oncogenes and tumour suppressors, which allows for a fine-tone regulation of PKM2 activity. The less active form of PKM2 drives glucose through the route of aerobic glycolysis, while active PKM2 directs glucose towards oxidative metabolism. Additionally, PKM2 possesses protein tyrosine kinase activity and plays a role in modulating gene expression and thereby contributing to tumorigenesis. We will discuss our current understanding of PKM2's regulation and its many contributions to tumorigenesis.

1. Introduction

Metabolism lies in the heart of cell biology. Understanding how cancer cells cope with metabolic needs for their unique biology has been a focus of cancer research for many years. It began with the landmark observation reported more than 80 years ago by Otto Warburg that cancer cells consumed more glucose and produced a large amount of lactate even in a well-oxygenized environment, a process known as aerobic glycolysis or the Warburg effect [1, 2]. While normal differentiated cells maximize ATP production by mitochondrial oxidative phosphorylation of glucose under normoxic conditions, cancer cells generate much less ATP from glucose by aerobic glycolysis. Despite being less efficient in ATP production, glycolysis is a much more rapid process [3, 4]. Cancers commonly deregulate pathways that enhance glycolysis, including activation of the PI3 K-ATK-mTOR pathway and upregulation of HIF-1 and c-Myc [5, 6]. The increase in aerobic glycolysis together with its dynamic process in cancer cells enables glycolytic intermediates to be redirected for the biosynthesis of cellular building blocks (nucleotides, amino acids, and lipids) while also producing ATP. Therefore, the Warburg effect/aerobic glycolysis meets the demands of cancer and proliferating cells for macromolecular synthesis and energy production [7, 8]. As a result, cancer cells display enhanced glucose uptake and produce higher levels of lactate [1, 2]. The Warburg effect was explored for the common clinical detection of tumors by fluorodeoxyglucose (2-deoxy-2-(^{18}F)fluoro-D-glucose) positron emission tomography (FDG-PET) [7]. In the glycolytic process, pyruvate kinase (PK) catalyzes the last reaction, transfer of a high-energy phosphate group from phosphoenolpyruvate (PEP) to ADP, producing ATP and pyruvate [9]. Pyruvate is then either reduced to lactate by lactate dehydrogenase (LDH) in the cytosol or enters the mitochondria to produce ATP through the tricarboxylic acid (TCA) cycle (Figure 1). Along the glycolysis pathway, intermediate metabolites can be channeled to synthesize amino acids, nucleotides, and lipids (Figure 1) if the rate of flux through the pathway is controlled. PK is an ideal candidate for this control [10] because (1) PK catalyzes the last reaction of the pathway (Figure 1) and (2) the reaction is essentially irreversible (Figure 1) [9, 11]. Therefore, lowering PK activity is expected to produce less pyruvate (Figure 1) or prevent complete

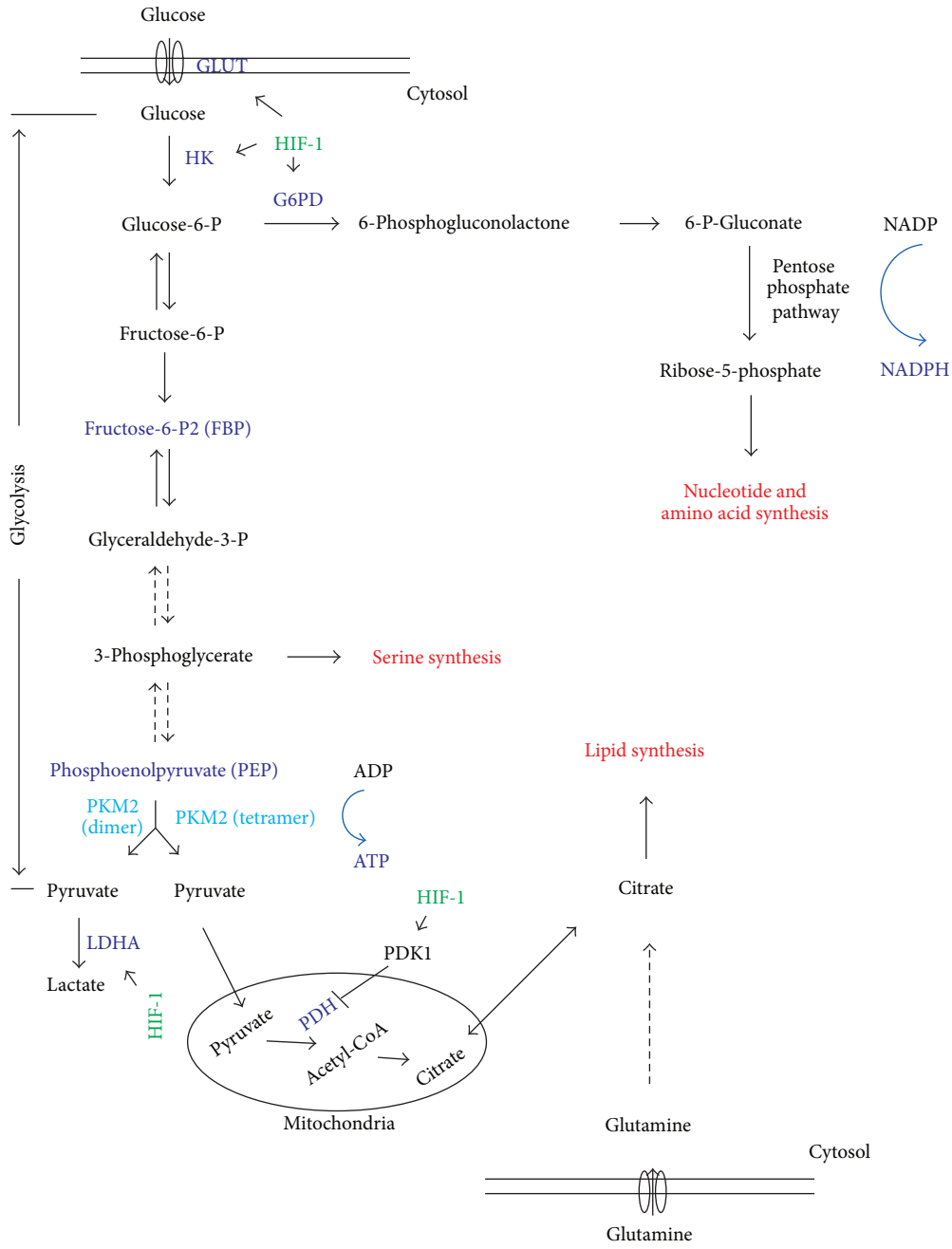

Figure 1: Schematic illustrating the cancer utilization of the metabolic pathways. Pyruvate kinase catalyzes the last step of glycolysis by converting PEP and ADP to pyruvate and ATP, respectively. PKM2 dimers and tetramers possess low and high levels of Pyruvate kinase activity, respectively. With reduced enzymatic activity, PKM2 dimer drives aerobic glycolysis, which allows the intermediate metabolites to be used for the synthesis of nucleotides, amino acids, and lipids and the production of reduced NADPH (see the pentose phosphate pathway). HIF-1 upregulates the indicated proteins. GLUT: glucose transporter, HK: hexokinase, G6PD: glucose-6-phosphate dehydrogenase, HIF-1: hypoxia-inducible factor 1, LDHA: lactate dehydrogenase A, PDK1: pyruvate dehydrogenase kinase isoenzyme 1, and PDH: pyruvate dehydrogenase.

conversion of glucose to pyruvate (1 molecule of glucose to 2 molecules of pyruvate). This enables the upstream glycolytic intermediates to accumulate and thus contribute to the shift of metabolism towards the anabolic phase for amino acids, nucleotides, and lipid production (Figure 1). Cancer cells explore this logic by predominantly using PKM2, an isoform of PK, as its activity can be dynamically regulated between the less active PKM2 dimer and the highly active PKM2 tetramer [12].

PK consists of four isoforms: the L (PKL) and R (PKR) isoforms encoded by the *PKLR* (1q22) gene and the M1 (PKM1) and M2 (PKM2) isoforms encoded by the *PKM2*

(15q23) gene. The *PKLR* gene is regulated by tissue-specific promoters. The full-length PKR isoform is expressed in red blood cells while the PKL isoform missing exon 1 is detected in liver and kidney [5, 13, 14]. PKM1 and PKM2 are produced from the *PKM2* gene by alternative splicing [15]. The highly active PKM1 is expressed in tissues that consistently need high levels of energy, like skeletal muscle, heart, and brain [5, 10]. PKM2 is expressed in most cells except adult muscle, brain, and liver [12, 16, 17] and is the predominant PK in proliferating and cancer cells [18]. While PKL, PKR, and PKM1 form stable tetramers (the active form of PK), PKM2 exists as both dimers and tetramers [18, 19]. The PKM2 dimer has a higher K_m towards PEP than the tetramer and thus is less active in converting PEP to ATP and pyruvate [19, 20]. While tetrameric PKM2 favors ATP production through the TCA cycle, dimeric PKM2 plays a critical role in aerobic glycolysis (Figure 1) [19]. Therefore, the dynamic equilibrium between dimer and tetramer PKM2 allows proliferating cells to regulate their needs for anabolic and catabolic metabolism. This not only explains why cancer cells predominantly express PKM2 but also reveals the existence of mechanisms that regulate this dynamic equilibrium. To ensure PKM2 expression, cancer cells also develop mechanisms for alternative splicing to produce PKM2 rather than PKM1. These mechanisms are regulated by oncogenes and tumor suppressors [21–25]. Surprisingly, dimeric PKM2 has additional functions in regulating gene expression in the nucleus [26].

2. PKM2 Contributes to Tumorigenesis

A large body of evidence supports the notion that cancers predominantly express PKM2 [14]. Immunohistochemical analysis revealed that PKM2 is commonly expressed in colon cancer [12], renal cell carcinoma (RCC) [27], and lung cancer [28]. PKM2 has been suggested to be a marker for RCC [29, 30] and testicular cancer [31]. Elevation of serum PKM2 levels was reported in patients with colon cancer [32], breast cancer [33], urological tumors [34], lung carcinoma, cervical cancer, and gastrointestinal tumor [18]. PKM2 was detected in the feces of patients with gastric and colorectal cancers [35]. Recently, mass spectrometry has demonstrated increases in PKM2, and the predominant presence of PKM2 was confirmed in RCC, bladder carcinoma, hepatocellular carcinoma, colorectal cancer, lung carcinoma, and follicular thyroid adenoma [16].

PKM2 expression correlates with tumorigenesis. High levels of PKM2 associate with poor prognosis for patients with signet ring cell gastric cancer [36]. A unique pattern of four expressed genes, including PKM2, was reported to predict outcomes for mesothelioma patients undergoing surgery [37]. Events that negatively impact tumorigenesis can also reduce PKM2 function. Vitamins K3 and K5 inhibit tumorigenesis along with potently inhibiting PKM2 activity [38]. Butyrate displays anticolon cancer effects along with the inhibition of PKM2 expression in neoplastic but not nontumor colon tissues [39]. Shikonin, a derivative of a Chinese herb with antitumor activities, induces necrosis and inhibits PKM2 expression in cancer cell lines [40]. A reverse correlation was observed between antitumor microRNA-326 and PKM2 in glioma [41]. Finally, the Spry2 tumor suppressor was reported to inhibit hepatocarcinogenesis via the MAPK and PKM2 pathways [42].

Furthermore, PKM2 possesses activities that directly promote tumorigenesis. Overexpression of PKM2 upregulates Bcl-xL in gastric cancer and promotes the proliferation and migration of colon cancer cells [43, 44]. Knockdown of PKM2 using specific siRNA inhibited cancer cell's proliferation and invasion in vitro and the formation of xenograft tumors in vivo [41, 45].

3. PKM2 Promotes Tumorigenesis via Regulating the Warburg Effect

The needs of energy production (ATP) and synthesis of cellular building blocks for proliferating cancer cells dictate the shift from oxidative to glycolytic metabolism even under normoxic conditions, the Warburg effect or aerobic glycolysis [2, 7, 8]. Under hypoxic conditions, cells metabolize glucose by anaerobic glycolysis, a process that is regulated by two master transcription factors, hypoxia-inducible factor (HIFs), and c-Myc [46]. Both transcriptional factors are also critical for aerobic glycolysis in cancer cells. Consistent with PKM2 being essential for aerobic glycolysis, a relationship exists among HIF-1, c-Myc, and PKM2. We will discuss the current understanding of these relationships.

3.1. Positive Feedback Regulation between PKM2 and HIF-1. It was first demonstrated by Christofk and colleagues in 2008 that knockdown of PKM2 in a panel of cancer cell lines decreased the rate of glycolysis and proliferation. Introducing PKM2 but not PKM1 to the knockdown cells not only enhanced glycolysis but also increased the ability to form xenograft tumors [12]. This research elegantly revealed that PKM2 is important and that the level of PK activity is essential, as the defects in PKM2 knockdown cells in supporting tumorigenesis could not be corrected by overexpression of the more active isoform PKM1. Furthermore, in comparison to PKM1 rescued cells, reintroducing PKM2 into knockdown cells rescued the deficiency of cell proliferation under hypoxic conditions.

This investigation also suggests that PKM2 may contribute to the adaptive response (hypoxia response) of cells to hypoxia, which is specifically relevant to tumorigenesis as solid cancers consistently face hypoxia intratumorally. It is thus a typical characteristic that cancers consistently execute hypoxia response. In the heart of this response lies the master transcription factor, hypoxia-inducible factor 1 (HIF-1) [47]. HIF-1 is a heterodimeric transcription factor, consisting of HIF-1α and HIF-1β. The β subunit is constitutively expressed, while the α subunit is directly regulated by oxygen (O_2) levels [48, 49]. Under normoxic conditions, HIF-1α is hydroxylated at prolines (P) 402 and 564 by three prolyl hydroxylase domain proteins (PHD1-3) in the presence of oxygen, α-ketoglutarate, iron, and ascorbate [50]. This results in the ubiquitination of prolyl-hydroxylated HIF-1α

by the von Hippel-Lindau (VHL) tumor suppressor and the subsequent degradation of HIF-1α [51, 52]. Under hypoxic conditions, HIF-1α is stabilized as a result of inhibiting prolyl hydroxylation, allowing HIF-1α to dimerize with HIF-1β in the nucleus. This leads to transcription of a set of genes to cope with reduced O_2 availability [53–55]. These target genes include those responsible for promoting glycolysis [56]. HIF-1 transactivates the glucose transporters GLUT1 and GLUT3, hexokinase (the first kinase in the glycolysis pathway), lactate dehydrogenase A (LDHA), and pyruvate dehydrogenase kinase 1 which phosphorylates and inhibits pyruvate dehydrogenase (PDH) [57] (Figure 1). Consistent with the Warburg effect's association with synthesis of cellular building blocks, HIF-1 also transactivates glucose-6-phosphate dehydrogenase (G6PD) to channel glucose-6-P into the pentose phosphate shunt for nucleotide and amino acid synthesis (Figure 1) [56]. Therefore, the collective actions of HIF-1 transcription activity seem to shift cells from oxidative metabolism to glycolysis (Figure 1). In line with these observations, PKM2 shares an intimate connection with HIF-1. The first intron of the *PKM2* gene contains the functional hypoxia-response element (HRE), thus also making it a target of HIF-1 [21].

PKM2 also possesses a positive feedback regulation towards HIF-1. PKM2 interacts with HIF-1α, a process that requires the prolyl hydroxylase 3 (PHD3). PHD3 binds to and causes hydroxylation of PKM2 at P303/408. This association and hydroxylation induces PKM2 to interact with HIF-1α, which plays a role in HIF-1-mediated transactivation of target genes including the *LDHA*, *PDK1*, and *VEGFA* (encoding the vascular endothelial growth factor) genes [21]. Additionally, PKM2 binds to p300 and enhances its recruitment to the HRE sites of HIF-1 target genes. Taken together, PKM2 functions as a HIF-1 coactivator by enhancing the Warburg effect in cancers [21, 22].

The regulation between HIF-1 and PKM2 also occurs under normoxic conditions, by changes in other signalling events which act to stabilize HIF-1α in cancer cells. HIF-1 is stabilized by mTOR and induced for degradation by VHL. Activation of mTOR is inhibited by tumor suppressors TSC1/TSC2 and facilitated by the PI3 K-AKT pathway [57]. Consistent with this knowledge, abnormal activation of the PI3 K-AKT-mTOR pathway and loss of function of tumor suppressors VHL, TSC1/2, and PTEN have been demonstrated to stabilize HIF-1α [57, 58]. Activation of mTOR by downregulation of TSC1/2 and PTEN induced PKM2 expression via stabilization of HIF-1α [59]. PKM2 makes essential contributions to mTOR-mediated aerobic glycolysis, as knockdown of PKM2 reduced glucose consumption and lactate production in cells with elevated mTOR activation. Furthermore, downregulation of PKM2 also suppressed mTOR-mediated tumorigenesis [59].

3.2. Positive Feedback Regulation between PKM2 and c-Myc. The *PKM2* gene produces both M1 and M2 isoforms through alternative splicing. The difference between these is the inclusion of exon 9 and exclusion of exon 10 for PKM1 and vice versa for PKM2 (Figure 2) [5, 15]. This mutually exclusive pattern of splicing is mediated by members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family, hnRNPA1, hnRNPA2, and hnRNP1/PTB (polypyrimidine track binding protein) [23, 60]. Binding of these proteins to the DNA sequence flanking exon 9 prevents its inclusion, resulting in the inclusion of exon 10 [23, 60, 61]. In order to achieve predominant expression of the M2 isoform, cancer cells have a strategy to preferentially splice the M2 isoform over M1 through c-Myc-mediated upregulation of hnRNPA1, hnRNPA2, and PTB (Figure 2) [23, 61]. This finding is supported by the discovery that cells with high levels of c-Myc activity also demonstrated high PKM2/PKM1 ratios [23, 62]. These observations are well in line with a large body of evidence indicating that c-Myc stimulates glycolysis and is required to coordinate with HIF-1 to regulate the cellular response to hypoxia [24, 46]. Thus, evidence suggests that PKM2 plays a role in c-Myc-mediated cancer metabolism and in c-Myc's communication with HIF-1. Adding to this attractive possibility is a recent demonstration that PKM2 also upregulates c-Myc transcription [63, 64], suggesting another positive feedback loop involving PKM2 in regulating the Warburg effect. Taken together, PKM2 is an integrated piece in the network of glycolysis regulation together with HIF-1 and c-Myc. The importance of hnRNPA1, hnRNPA2, and PTB in splicing PKM2 has also been explored by tumour suppression activity. The microRNAs mir-124, mir-137, and mir-340 inhibit colorectal cancer growth by repressing the expression of these hnRNAs favouring PKM1 splicing, thereby inhibiting aerobic glycolysis or the Warburg effect [65].

4. Regulation of PKM2 in the Warburg Effect during Tumorigenesis

Cancers have developed a complex regulation of PKM2 to meet the needs for energy and synthesis of nucleotides, amino acids, and lipids. These mechanisms center on regulating PKM2's expression, allosteric regulation, and modifications. The latter two mechanisms directly or indirectly affect PKM2 activity through physical interaction and by regulating the PKM2 dimer-tetramer dynamic.

4.1. Transcription Regulation. In addition to the above discussion of HIF-1 and c-Myc-mediated transcription and splicing of PKM2, transcription of the *PKM2* gene is also regulated by the SP1 and SP3 transcription factors [5, 22, 66]. The network of PI3 K-AKT-mTOR (mammalian target of rapamycin) plays a critical role in cell metabolism, proliferation, and survival and is one of the most frequently activated pathways in cancer owing to the activation of kinases and the inactivation of tumor suppressors, TSC1/2 (tuberous sclerosis 1/2) and PTEN [67]. Nutrient status is well known to modulate mTOR activation [68]. Under normoxic conditions, mTOR activity induces PKM2 expression through the combination of HIF-1α and c-Myc [59, 69]. Inhibition of mTOR has been found to reduce glycolysis and PKM2 expression [70]. Elevation in PTEN function reduces glucose uptake and the Warburg effect and inhibits PKM2 expression [25]. In a feedback

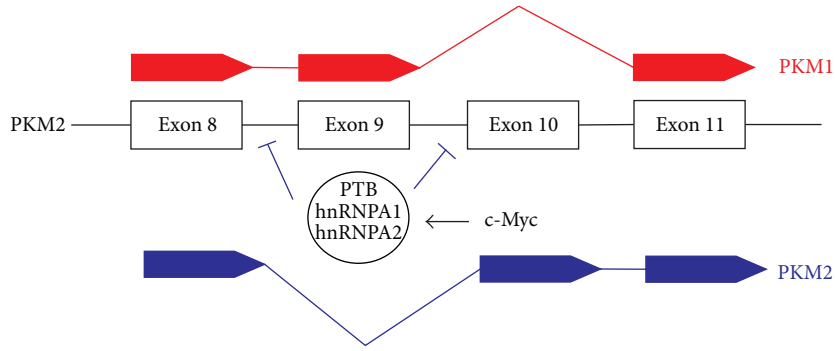

Figure 2: Schematic illustration of alternative splicing of PKM1 and PKM2. The proportion of the *PKM2* gene is shown. c-Myc upregulates the indicated complex which inhibits the splicing for exon 9, resulting in its exclusion in PKM2. PTB: polypyrimidine track binding protein; hnRNPA1 and hnRNPA2: heterogeneous nuclear ribonucleoprotein 1 and 2.

manner, PKM2 is able to sustain mTOR activation in serine-depleted medium by enhancing endogenous serine synthesis [71]. Taken together, evidence supports that the upregulation of PKM2 plays an important role in the mTOR-mediated Warburg effect in tumors.

4.2. Regulation of the Dimer-Tetramer Dynamics. Tumor cells express high levels of dimer PKM2 [14, 32]. Among the four PK isoforms, PKM2 is the only one to be allosterically regulated between a less active dimer and an active tetramer [18, 19]. These different forms of PKM2 regulate glucose metabolism through either the TCA cycle or glycolysis. Accumulating evidence supports the concept that the less active PKM2 dimer drives aerobic glycolysis, while the active PKM2 tetramer produces pyruvate for oxidative phosphorylation (Figure 1) [12, 72–74]. PKM2 is regulated by fructose-1,6-biphosphate (FBP), an upstream intermediate of glycolysis which when bound to PKM2 activates tetramerization through high affinity association [75–77]. Binding of tyrosine-phosphorylated peptides dissociates FBP from the PKM2 tetramer, resulting in conversion to the PKM2 dimer [72]. The less active PKM2 dimer is critical in mediating aerobic glycolysis in tumor cells based on high levels of lactate production and lower oxygen consumption [72]. Disrupting the binding of the phosphotyrosine peptide in a PKM2 mutant (M2KE) increased PKM2 kinase activity, which was associated with reduction in lactate production and elevation of oxygen consumption [72]. In supporting the low levels of cellular pyruvate kinase activity being critical for aerobic glycolysis, replacing PKM2 with PKM1 led to an increase in cellular pyruvate kinase activity, decreasing lactate production and elevating oxygen consumption [12, 74].

Collectively, evidence supports that the PKM2 dimer is critical in mediating aerobic glycolysis. In addition to the above mechanism regulating PKM2 activity, PKM2 was also controlled by tyrosine phosphorylation [73]. It was observed in 1988 that PKM2 was tyrosine-phosphorylated in v-Src-transformed chicken embryo cells. This phosphorylation reduced the affinity of PKM2 towards its substrate phosphoenolpyruvate (PEP) [78]. In vitro, v-Src was able to directly phosphorylate PKM2 [78]. Although this investigation suggested that v-Src phosphorylated PKM2, the sites of phosphorylation remain unknown. Recent development demonstrated that PKM2 was phosphorylated at several tyrosine residues, including Y105, by fibroblast growth factor receptor type 1 (FGFR1) [73]. Phosphorylation at Y105 causes FBP to dissociate from the PKM2 tetramer, which results in PKM2 dimers and promotes the Warburg effect based on the production of lactate [73]. Conversely, abolishing Y105 phosphorylation by substitution with phenylalanine (Y105F) elevated the kinase activity, resulting in decreased lactate production and increased oxygen consumption [73]. Taken together, evidence demonstrates that phosphorylation at Y105 plays a role in the conversion of PKM2 tetramers to dimers.

More importantly, regulation of PKM2 dimer and tetramer conversion is critical for tumorigenesis. While the less active PKM2 dimer enhances xenograft tumor formation, enforced formation of active PKM2 (KE and Y105F mutations) and replacing PKM2 with PKM1 inhibited the formation of xenograft tumors [72, 73, 79]. In line with this concept, the conversion between dimer and tetramer PKM2 is also used in tumour suppression to inhibit tumorigenesis. The death-associated protein kinase (DAPK) tumor suppressor activates PKM2 by stabilizing the PKM2 tetramer via a direct association. This reduces cancer metabolism or the Warburg effect, which may be one aspect of DAPK-mediated tumor suppression [80, 81].

In line with these observations, several small molecule PKM2 activators have been identified. Among them, DASA-58 (the substituted N, N′-diarylsulfonamide NCGC00185916) and TEPP-46 (the thieno-[3,2-b]pyrrole [3,2-d]pyridazinone NCGC00186528) activate PKM2 by inducing PKM2 tetramerization. Unlike FBP-induced activation, the tetramer induced by these compounds is resistant to tyrosine-phosphorylated peptide-mediated conversion to the PKM2 dimer. This suggests that FBP and these small molecule activators bind PKM2 at distinct sites, but, importantly, all inhibit tumorigenesis [74, 82, 83]. Additionally, a new set of chemical platform bases, the quinolone sulfonamide-based PKM2 activators, have recently been reported. Similar to DASA-58 and TEPP-46, these activators also stabilize the PKM2 tetramer via binding to a pocket distinct from FBP

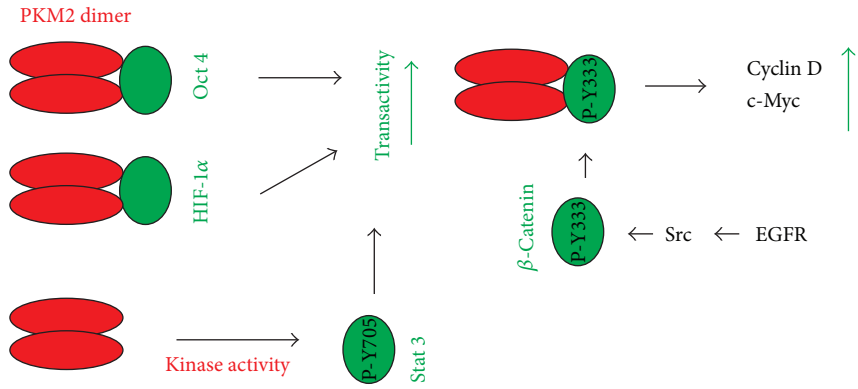

Figure 3: Diagram showing the nuclear function of PKM2. PKM2 dimers in the nucleus bind to Oct 4 and HIF-1α and enhance their transcription activity; EGFR signal activates Src tyrosine kinase, which phosphorylates β-catenin at tyrosine (Y) 333 (P-Y333). PKM2 binds Y333-phosphorylated β-catenin, contributing to β-catenin-mediated transcription of cyclin D and c-Myc; PKM2 dimer possesses kinase activity that phosphorylates Stat 3 at Y705, which enhances Stat 3's transcriptional activity.

binding and thus prevent the PKM2 tetramer from tyrosine-phosphorylated peptide-mediated disruption. Quinolone sulfonamide-based PKM2 activators reduce carbon flow towards the serine biosynthetic pathway, rendering cells to serine auxotrophy [84].

4.3. Factors Affecting PKM2 Activity via Physical Association. In addition to the above two small molecule PKM2 activators, a third activator was recently reported by the same research group based on modifications to one of their previous compounds [85]. The mechanism underlying this activation remains to be defined. A series of PKM2 activators (1-(sulfonyl)-5-(arylsulfonyl)indoline) were also reported very recently [86]. In contrast to these, potent small molecule PKM2 inhibitors which may in part induce cell death by inhibiting PKM2 activity have also been developed [87]. Furthermore, the pyruvate kinase activity of PKM2 can be inhibited by association with several distinct proteins. While the nuclear promyelocytic leukemia (PML) protein functions as a tumor suppressor, cytosolic PML was reported to specifically inhibit tetrameric but not dimeric PKM2 activity, thereby contributing to the Warburg effect [88]. Prolactin signal promotes cell proliferation by inducing its receptor to associate with PKM2, leading to PKM2 activity reduction [89]. The MUC1-C oncoprotein was reported to promote breast cancer tumorigenesis in part via inhibiting PKM2 activity. Although interaction of MUC1-C Cys3 with PKM2 C-domain Cys474 results in activation of PKM2, oncogenic signals from EGFR (epidermal growth factor receptor) can alter the association of MUC1-C and PKM2, thereby leading to inhibition of PKM2 activity [90]. EGFR phosphorylates MUC1-C at tyrosine 46, causing MUC1-C to interact with PKM2 at Lys433. This association inhibits tetrameric PKM2 activity and thereby increases aerobic glycolysis along with glucose uptake [90]. PKM2 was also found to interact with human papillomavirus 16 (HPV16) protein E7, which may contribute to HPV16-induced cervical cancer [91]. A potential therapeutic protein TEM8-Fc, consisting of a portion of the tumor endothelial marker 8 (TEM8) and the Fc domain of human IgG1, was found to associate with PKM2 [92]. Whether this interaction contributed to TEM8-Fc-associated tumor suppression was not clear [92]. Consistent with the knowledge that PKM2 plays a critical role in regulating aerobic glycolysis and biosynthesis for cellular building blocks, PKM2 is activated by serine but inhibited by alanine and phenylalanine when bound to these amino acids [93].

4.4. Posttranslational Modifications of PKM2. A reduction in activity was reported by acetylation of PKM2 at lysine (K) 305 in response to high levels of glucose. This modification reduces PKM2 activity and its affinity towards the PEP substrate, resulting in PKM2 degradation via chaperone-mediated autophagy [94]. As a result, acetylation enhances cell proliferation by increasing the availability of glycolytic intermediates for anabolic synthesis [94, 95].

PKM2 also plays a role in cell survival to oxidative stress. Acute increases in intracellular levels of ROS (reactive oxygen species) induce oxidation of PKM2 at Cys358. This reduces PKM2 activity, which allows the accumulation of glucose-6-phosphate and thus shifts glucose flux through the pentose phosphate pathway (PPP) to generate reduced NADPH (Figure 1). As PPP is the major pathway of generating reduced NADPH, oxidation-mediated inhibition of PKM2 is therefore a mechanism of detoxification during oxidative stress. Consistent with this notion, substitution of C358 with S358 to produce oxidation-resistant mutants sensitized cells to oxidative stress and inhibited xenograft tumor formation [96]. A similar antioxidative stress function of PKM2 is also mediated through binding to CD44, a major cell adhesion molecule. Cancer stem cells are known to be CD44 positive, so this interaction is consistent with CD44 promoting cancer progression, metastasis, and chemoresistance [97, 98]. CD44's tumorigenic function is in part also attributable to its association with EGFR [99]. Consistent with these observations, PKM2 was reported to bind CD44, resulting in receptor tyrosine kinase-mediated phosphorylation of PKM2 and inhibition of PKM2 activity. This enhanced glucose flux

through the PPP pathway to generate reduced NADPH and counteract oxidative stresses through detoxification [61, 100].

5. The Nuclear Function of PKM2

PKM2 displays intriguing nonglycolytic functions in the nucleus. In addition to its cytoplasmic presence to regulate aerobic glycolysis, PKM2 was also detected in the nucleus in response to interleukin-3 and apoptotic signals [101, 102]. Nuclear PKM2 binds Oct 4 through its C-terminal region (residues 307–531), enhancing Oct-4-mediated transcription [103] (Figure 3). Nuclear PKM2 was also reported to be a coactivator of HIF-1 [21] (Figure 3). EGFR signaling was reported to activate Src tyrosine kinase, which in turn phosphorylates β-catenin at Y333. PKM2 binds to tyrosine-phosphorylated β-catenin in the nucleus and contributes to β-catenin-mediated transactivation of cyclin D and c-Myc, thereby promoting both cell proliferation and tumor progression (Figure 3). This process requires the kinase activity of PKM2 [63, 104]. Since the binding of tyrosine-phosphorylated peptides maintains PKM2 in its dimer status [72], these observations suggest that dimerized PKM2 binds and enhances β-catenin function, in which a new kinase activity rather than pyruvate kinase activity might be involved. Indeed, it was very recently reported that the PKM2 dimer contributes to its nuclear function and possesses protein tyrosine kinase activity. Surprisingly, instead of using high-energy ATP, PKM2 uses the high-energy phosphate from PEP as a phosphate donor to phosphorylate its protein substrates [26]. The PKM2 dimer phosphorylates Stat 3 at Y705 in the nucleus and thus enhances Stat 3 transcription activity [26, 105] (Figure 3). Taken together, while tetramer PKM2 is a pyruvate kinase, dimer PKM2 can also act as a protein tyrosine kinase [26].

6. Concluding Remarks and Future Perspectives

The last decade has seen a high reemergence of interest in the Warburg effect, the typical cancer cell metabolism that was reported almost 90 years ago. The detailed molecular and genetic knowledge accumulated in the last few decades of extensive cancer research has rapidly advanced our understanding of cancer metabolism. Mutations in several enzymes of the TCA-cycle were discovered, including isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2), succinate dehydrogenase (SDH), and fumarate hydratase (FH) [106–109]. These mutations collectively reduce TCA-cycle-mediated oxidative phosphorylation, resulting in an accumulation of metabolites for the biosynthesis of amino acids, nucleotides, and lipids as well as increases in glucose uptake [110]. The increases in glucose uptake together with aerobic glycolysis yield a robust elevation of lactate production. Although recent development suggests that the by-product of aerobic glycolysis (lactate) contributes to overall tumorigenesis [111, 112], it is also critical for cancer cells to efficiently export lactate to maintain the flux of glycolysis and to prevent cellular acidification [112]. Cancer cells accomplish this task in part by upregulation of the monocarboxylate transporters (MCTs) [112]. Another strategy to reduce the cellular burden of lactate accumulation during aerobic glycolysis may be the prevention of a complete conversion of glucose to lactate (1 glucose for every 2 lactate molecules) by reducing the conversion of PEP to pyruvate. This would allow the glycolytic intermediates to be used for macromolecular synthesis. Therefore, predominantly using the less active PKM2 dimer fits this logic.

Accumulating evidence obtained in the last 10 years demonstrates that PKM2's glycolytic enzyme activity is regulated by oncogenes and tumor suppressors [21–25]. These regulations center on modulation of aerobic glycolysis. Favoring a shift of the dimer-tetramer dynamic towards dimerization is critical for PKM2 to promote the Warburg effect, leading to cell proliferation and tumorigenesis.

Surprisingly, in addition to its glycolytic pyruvate kinase activity in the cytosol, the PKM2 dimer also displays protein tyrosine kinase activity in the nucleus and nuclear PKM2 promotes the transcriptional activities of HIF, β-catenin, STAT 3, and Oct 4 [21, 26, 63, 103–105]. This all indirectly contributes to cancer metabolism and other aspects of tumorigenesis. In light of this new development, future research should determine the contributions of the cytosolic versus nuclear PKM2 dimer to aerobic glycolysis.

Effort is currently underway to target PKM2 for cancer therapy, which is part of the current attempt in targeting cancer metabolism. Several small molecule PKM2 inhibitors and activators have been developed [61]. As nearly complete knockdown of PKM2 does not completely inhibit cancer cell proliferation, the utility of PKM2 inhibition in targeting cancer should be cautious [61]. On the other hand, small molecule activators might be an attractive approach. However, several factors call for precautions in targeting PKM2. (1) PKM2 is also expressed in normal tissue [16, 17] and the function of PKM2 in normal tissues has not yet been determined; (2) genetic changes in PKM2 have not been reported in primary cancers; (3) despite modulation of PKM2 which affects formation of xenograft tumors, whether tissue-specific manipulation of PKM2 impacts tumorigenesis is still on the waiting list; (4) as PKM2 was detected in cancer stroma [113, 114], whether it plays a role in tumorigenesis by affecting cancer-associated fibroblasts is not clear; (5) while aerobic glycolysis has been a hot topic in the last decade, its impact on cancer stem cells (CSCs) has not been addressed. As it is becoming increasingly clear that CSCs play a critical role in tumorigenesis, especially in tumor progression and metastasis [115], it would appear critical to understand whether targeting cancer metabolism in general and PKM2 in particular will have an inhibitory effect on CSCs. This knowledge became important as it was suggested that glioma CSCs (GSCs) may not use aerobic glycolysis to the same degree as differentiated cancer cells. Thus, targeting PKM2 or cancer metabolism may still spare GSCs [116].

Acknowledgments

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Review Article

Targeting Metabolism to Induce Cell Death in Cancer Cells and Cancer Stem Cells

Claire Pecqueur,[1,2,3] Lisa Oliver,[1,2,4] Kristell Oizel,[1,2] Lisenn Lalier,[1,2,5] and François M. Vallette[1,2,5]

[1] *Equipe Labellisée Ligue Contre le Cancer, Equipe 9 CRCNA, INSERM UMR 892, CNRS UMR 6299, 44007 Nantes, France*
[2] *Faculté de Médecine, Université de Nantes, 44007 Nantes, France*
[3] *Centre de Recherche en Cancérologie Nantes Angers, INSERM UMR 892, CNRS UMR 6299, Université de Nantes 8 Quai Moncousu BP 70721, 44007 Cedex 1 Nantes, France*
[4] *Centre Hospitalier, Universitaire (CHU) de Nantes, 44007 Nantes, France*
[5] *Institut de Cancérologie de l'Ouest, René Gauducheau, Saint-Herblain, 44805 Nantes, France*

Correspondence should be addressed to Claire Pecqueur; claire.pecqueur@univ-nantes.fr

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Abnormal metabolism and the evasion of apoptosis are considered hallmarks of cancers. Accumulating evidence shows that cancer stem cells are key drivers of tumor formation, progression, and recurrence. A successful therapy must therefore eliminate these cells known to be highly resistant to apoptosis. In this paper, we describe the metabolic changes as well as the mechanisms of resistance to apoptosis occurring in cancer cells and cancer stem cells, underlying the connection between these two processes.

1. Introduction

Cell proliferation involves the replication of all cellular contents with the required energy for this to happen. In normal cells, glucose participates in cellular energy production through glycolysis as well as through its complete catabolism via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). In addition to glucose, glutamine is also required to feed the TCA cycle. Lipids, amino acids, and nucleotides necessary for the biosynthesis of the daughter cells are mostly provided by intermediate metabolites of these pathways. To prevent aberrant cell proliferation, these pathways are tightly regulated. However, cancer cells overcome these controls, in particular by acquiring genetic mutations leading to the activation of oncogenes (pten, myc) or loss of tumor suppressors (p53) [1]. For example, a major regulator of metabolism is phosphoinositol 3 kinase (PI3K). PI3K is activated by growth factors resulting in, among others, the activation of Akt and mTOR. This activation is necessary for both cell proliferation as well as glucose uptake and use. In addition to its role in glucose metabolism, this pathway also regulates the redirection of free amino acids to protein synthesis via the mTOR-signaling pathway.

2. Metabolic Modifications in Cancer Cells

In contrast to normal cells, most cancer cells predominantly produce energy by a high rate of glycolysis followed by lactate fermentation, even in the presence of oxygen, a less efficient metabolism compared to a low rate of glycolysis followed by mitochondrial oxidation of pyruvate [2]. Typically, rapidly proliferating tumor cells have glycolytic rates up to 200 times higher than those of their normal tissue of origin, even in the presence of oxygen [3]. This observation resulted in the development of 2-[18F]-fluoro-2-deoxy-D-glucose positron emission tomography (PET) to detect glucose uptake and lactate production for tumor imaging.

Pyruvate, which is at the crossroad between lactate production and OXPHOS, constitutes a key metabolic intermediate. In normal cells, the fate of pyruvate depends on many factors, one of which is oxygen availability. In the presence

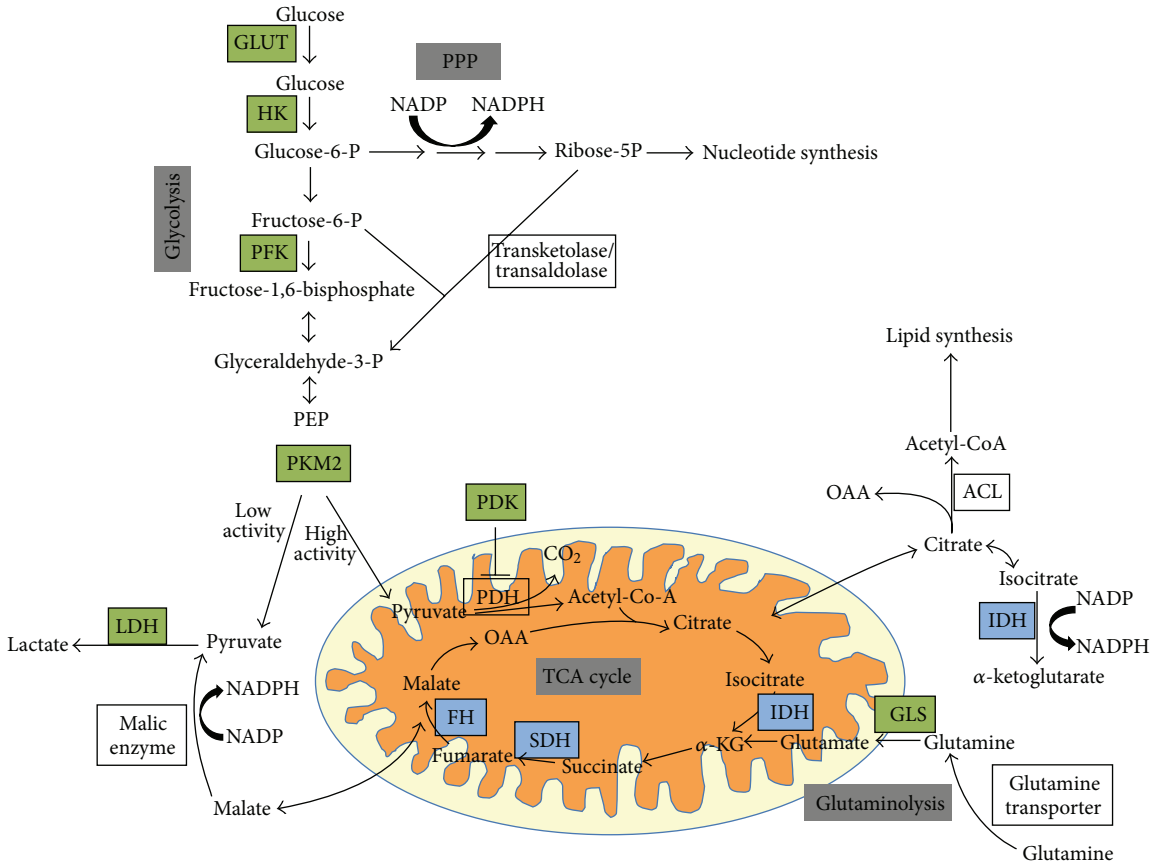

Figure 1: Metabolic adaptations of cancer cells. Glucose and glutamine are the 2 major substrates used by cancer cells. Glucose is imported into the cells through glucose transporters (GLUT) where it is phosphorylated by Hexokinase (HK). It will then be either metabolized through glycolysis or diverted to the pentose phosphate pathway (PPP). Glucose-derived pyruvate is mainly converted into lactate in cancer cells instead of being imported into mitochondria to be oxidized in acetyl CoA to support mitochondrial energy production. MYC enables cancer cells to maximize glutamine uptake from the extracellular space through the upregulation of the glutamine transporter. Once glutamine enters the cell, it can be metabolized through glutaminolysis to provide glutamate. The transamination of glutamate to αKG will feed the TCA cycle (adapted from Vander-Heiden et al. [9]). αKG: α-KetoGlutarate; TCA: tricarboxylic acid cycle; PDH: pyruvate dehydrogenase; LDH: lactate dehydrogenase; PDK: PDH-kinase; PK: pyruvate kinase; PEP: phosphoenolpyruvate; GLS: glutamine synthase; SDH: succinate dehydrogenase; FH: fumarate hydratase; 2-HG: 2D-hydroxyGlutarate; IDH: isocitrate dehydrogenase; HK: hexokinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

of oxygen, the pyruvate is directed into mitochondria to be converted into acetyl CoA by the pyruvate dehydrogenase (PDH) or into alanine by transamination. Inside the mitochondria, pyruvate is completely oxidized through the TCA cycle, feeding reductive equivalents to the electron transport chain. When oxygen is limited, as in muscles that have undergone prolonged exercise, pyruvate is not consumed in the TCA cycle but is rather converted into lactic acid by lactate dehydrogenase (LDH) in a process termed anaerobic glycolysis. In contrast, cancer cells shift their metabolism toward lactate production even in the presence of oxygen [4], partly through genetic modifications that stabilize the transcription factor Hypoxia Inducible Factor (HIF) involved in the adaptation of the cells to hypoxia, under nonhypoxic conditions as well as generating an adaptive response to the hypoxic microenvironment (Figure 1). By stimulating the expression of glucose transporters and glycolytic enzymes, HIF-1 promotes glycolysis to generate more pyruvate [5]. Furthermore, HIF-1 actively limits the mitochondrial consumption of pyruvate at two levels: (i) through the enzyme PDK (PDH-kinase), which in turn inhibits PDH activity preventing the conversion of pyruvate into acetyl CoA, and thereby limiting mitochondrial metabolism of pyruvate [6] and (ii) through the direct activation of LDH [7, 8]. Overall, these processes allow the regeneration of NAD^+ required for ATP production through glycolysis.

Pyruvate synthesis by pyruvate kinase (PK) is modified in cancer cells. This step is highly regulated by the type of isoform expressed and/or by allosteric regulation. Four isoforms have been described with a specific tissue distribution. PKL is found in the liver and kidney and PKR in red blood cells. The two isoforms PKM1 and PKM2 are different splicing products of the same gene [10]. PKM1 is expressed in organs with high energy demands such as muscle and brain while PKM2 is expressed in differentiated tissue such as lung, fat, and pancreatic islets as well as in all cells with a high rate of nucleic

acid synthesis such as proliferating cells, embryonic cells, and, especially, tumor cells [11]. In contrast to PKM1, which exists in a constitutively tetrameric active form, PKM2 exists under dimeric and tetrameric forms. The dimeric PKM2, which is inactive, results in an accumulation of upstream glycolytic intermediates, thus favoring their redistribution towards other biosynthetic pathways (synthesis of nucleic acids, phospholipids, or amino acids). PKM2 is different from other pyruvate kinase isoforms because it can bind to proteins that are phosphorylated on tyrosine residues in response to cell growth signals. This phosphotyrosine-binding activity negatively regulates the enzymatic activity providing a link between cell growth signals and the regulation of glycolysis. Thus, the ratio tetramer: dimer of PKM2 determines whether carbons from glucose are converted into lactate via pyruvate or channeled into building block synthesis (Figure 1). This ratio depends mainly on the availability of fructose-1,6-phosphate (FBP) since high concentrations of this enzyme induce the association of dimeric forms into tetramers, which in turn leads to lactate production with energy regeneration (Warburg effect) until the level of FBP is reduced and the tetramer dissociates into dimers.

Besides glycolysis, another metabolic pathway used by cancer cells to provide macromolecules is glutaminolysis, which generates reductive equivalents such as NADPH by replenishing the TCA cycle [4]. Glutamine is a conditional amino acid in the sense that, under normal conditions, it can be synthesized in most cells. However, during rapid growth, the cellular demand exceeds its supply and glutamine becomes essential. Glutamine provides energy through the TCA cycle as well as nitrogen, sulfur, and carbon skeletons for proliferating cells. Tumor cells tend to have a large pool of glutamate, and this pool is maintained by their ability to convert glutamine into glutamate through glutamine synthase (GLS), a mitochondrial enzyme highly active in tumors (Figure 1). In fact, limiting GLS activity results in a decreased growth rate in tumor cells both *in vitro* and *in vivo* [12]. Glutamate is also a nitrogen carrier for alanine and aspartate synthesis through the activity of aminotransferase. Alanine is used in protein synthesis and is also avidly secreted by tumor cells while aspartate contributes to the synthesis of proteins and nucleotides as well as feeding the electron transport chain via the malate/aspartate shuttle. Glutamine is also involved in the biosynthesis of glutathione, one of the major antioxidant molecules of the cells. In tumors, maintaining the reduced form of glutathione is crucial for cell survival since it allows the cell to resist oxidative stress associated with a rapid metabolism, DNA-damaging agents, or inflammation [13].

Beyond its roles in intermediary metabolism, glutamine exerts other effects that support cell survival and growth [10, 14]. Reflecting the importance of glutamine in anabolic metabolism, cells have developed glutamine-dependent mechanisms to control growth, including the modulation of signal transduction pathways. For example, a recent study showed that cellular uptake of glutamine and its subsequent rapid efflux in the presence of essential amino acids is required for the activation of the mTOR pathway [15]. Glutamine uptake is regulated by SLC1A5, and its loss inhibits cell growth and activates autophagy. Other data have identified a role for glutamine in the extracellular signal-regulated protein kinase- (ERK-) signaling pathway. This has been best characterized in intestinal epithelial cells, which consume glutamine as their major bioenergetic substrate and require glutamine for both proliferation and survival. In these cells, the addition of glutamine was sufficient to stimulate ERK signaling whereas glutamine deprivation was associated with increased apoptosis [16].

The interplay between glutamine and glucose utilization would depend on the particular oncogene/tumor suppressor involved in tumor progression. While the myc oncogene induces both aerobic glycolysis and glutaminolysis, activated β-catenin induces glutamine synthesis. However, glutamine synthetase is not highly expressed in all tissues, and thus glutamine consumption and addiction are dependent on the metabolic profile of the cancer cells. In addition, it has been postulated that ammonia, a byproduct of glutamine metabolism, is a diffusible activator of autophagy [17].

Finally, the tumor microenvironment containing supporting host cells (stroma, adipocyte, fibroblasts, muscle, and endothelial cells) and immune cells plays an important role in tumor initiation, tumor progression, and in the response of tumoral cells to therapy. Several recent publications have highlighted a metabolic crosstalk called the "reverse Warburg effect" where aerobic glycolysis in host stromal cells fuels anaplerotic metabolism in tumor cells [18]. In this two-compartment model, the anabolic tumor cells obtain energy from the surrounding host cells by inducing catabolic processes such as autophagy, mitophagy, and aerobic glycolysis, which would result in the overproduction of high-energy metabolites such as L-lactate, ketone bodies, and glutamine [19]. These metabolites are taken up by tumor cells and converted into acetyl CoA, which enters the TCA cycle resulting in the production of ATP. In addition, as a result of the enhanced glycolytic metabolism in the tumor, lactate accumulates in the tumor microenvironment. Besides the role of lactate in metastases [20, 21], this acidification plays an important role in tumor immunosuppression since lactate has been shown to inhibit the differentiation and/or the function of immune cells [22, 23].

3. Genetic Mutations in Metabolic Enzymes and Cancer

Although most cancer cells have functional mitochondria, a subset of human tumors harbors mutations that impair mitochondrial metabolism [24]. Two major classes of mutations occur in genes required for the function of the TCA cycle enzymes namely the succinate dehydrogenase (SDHA, SDHB, SDHC, SDHD, and SDHAF2) and the fumarate hydratase (*FH*) [25, 26]. Heterozygous germline mutations in SDHB, SDHC, and SDHD subunits were identified in paragangliomas and pheochromocytomas whereas germline mutations in FH predispose to renal cell cancer. In all cases, the loss of function mutations are followed by a somatic "second hit" resulting in the loss of the other allele in tumor cells. These mitochondrial enzymes catalyze, respectively, the conversion of succinate into fumarate and the

reversible conversion of fumarate into malate in the TCA cycle (Figure 1). The loss of function of SDH and FH results in the accumulation of succinate and fumarate in the cytosol. The accumulation of succinate and fumarate impairs the enzymatic activity of several αKG-dependent dioxygenases including the PHDs (Prolyl-Hydroxylases). These proteins which are regulated by changes in the oxygen concentration initiates the hydroxylation of HIF-1α resulting in the ubiquitination /degradation of the α-subunit of HIF 1 under normoxia. However, only PHD2 has been shown to directly interact with HIF-1α. Similarly, fumarate inhibits PHD-2 activity leading to HIF-1α stabilization. A recent analysis shows that 12% of glioblastoma multiforme (GBMs), the most common and most aggressive malignant brain tumor, have a mutation in the gene-encoding isocitrate dehydrogenase-1 (IDH-1) [27]. This mutation is present in more than 90% of recurrent GBMs while it is present in less than 5% of *de novo* GBMs [28]. Mutations in IDH-1 and IDH-2 (isocitrate dehydrogenase-2) have also been identified in acute myeloid leukemia [29]. These enzymes catalyze the conversion of isocitrate into αKG and as such play important roles in metabolism and growth (Figure 1). IDH mutations are associated with a neomorphic activity of the enzyme leading to the production of an oncometabolite, 2D-hydroxyglutarate (2-HG) [30]. 2-HG accumulation impairs DNA methylation via the inhibition of αKG-dependent dioxygenases that carry out diverse functions such as prolyl-hydroxylation, histone demethylation, and epigenetic modifications of DNA [31, 32]. The expression of this mutation also impairs hematopoietic and adipocyte differentiation [33–35].

Finally, a common cellular response to impaired mitochondrial metabolism, for example, in cells deficient in FH, is the glutamine-dependent reductive carboxylation [36]. During this process, αKG is carboxylated by IDH isoforms to generate isocitrate, which in turn generates citrate, oxaloacetate (OAA), and acetyl CoA. The latter is crucial for fatty acids synthesis and protein acetylation while OAA is reduced to malate [36, 37]. This mechanism would enable cells with an impaired OXPHOS to maintain cell proliferation.

4. Metabolic Modification in Cancer Stem Cells

The cancer stem cell concept was proposed several decades ago to explain two recurring observations. First, most cancers consist of phenotypically heterogeneous tumor cells, and, second, only a fraction of cells from both hematologic and solid tumors are tumorigenic [38–40]. Later, it was established that the tumorigenic potential was not equally shared by all cells within an individual tumor but restricted to a distinct subset. Thus, tumors are made up of a large subset of cells with a high rate of division unable to give rise to a new tumor and a small number of cells with a slow rate of division supplying the tumor with new tumor initiating cells. Two models could explain this tumor heterogeneity. The stochastic model predicts that tumors are biologically homogeneous and the behavior of the cancer cells is influenced by intrinsic or extrinsic factors resulting in a heterogeneity in the expression of cell markers, cell cycle, or in tumor initiation ability. In contrast, the hierarchic model predicts that tumors are organized as a normal tissue with stem cells maintaining the tissue hierachy [41, 42]. These CSCs were identified for the first time in acute myeloid leukemia [43]. They were described as an unusual and small population of cells (0.01–1% of the total population), capable of inducing leukemia after serial transplantation into immunodeficient mice. CSCs were subsequently identified in numerous solid tumors. In breast tumors, a population of cells enriched in markers $CD44^+$ $CD24^{-/low}$ was identified as CSCs [44]. More recently, CSCs have been described in brain tumors [45], medulloblastoma ependymoma [46], colorectal tumors [47], pancreas [48], ovarian [49], liver [50], prostate [51], lung [52], and in melanomas [53]. Like normal stem cells, CSCs reside in niches, that is, a microenvironment capable of maintaining a balance between self-renewal and differentiation. However, all tumors do not seem to follow the model suggested by the presence of CSCs. Indeed, some tumors have little heterogeneity and seem to follow a model of clonal evolution or a stochastic model, in which a population of proliferating cells gives rise to the tumor [54]. Nevertheless, both models are not mutually exclusive. Indeed, the CSCs may undergo clonal evolution and become more aggressive due to mutations or epigenetic modifications. This phenomenon has been described in leukemia [55] and has also been observed in the case of serial transplantation in animals, which generate more aggressive tumors [56]. Finally, a controversy exists about whether these CSCs are derived from normal stem cells that have transformed or cancer cells that have dedifferentiated.

At present, we do not know if CSCs come from normal stem cells or more differentiated cells that have acquired dedifferentiating mutations. One hypothesis for the existence of CSCs suggests that these cells derive from normal stem cells that have acquired mutations that allow them to escape the control of the niche. Another hypothesis is that dysregulation of growth factors secreted by the niche could lead to uncontrolled proliferation of stem cells and, as a result, tumorigenesis [57].

Cancer stem cells have been defined, by analogy to normal stem cells, in that they are capable of self-renewal and can generate all the differentiated cells found within the tumor [45, 58]. One feature of CSCs is their ability to expel chemicals, most often lipophilic, via membrane transporters (multidrug resistance proteins: MDR). Thus, in cancer cell cultures grown in the presence of Hoechst 33342 (a DNA intercalant), a portion of the cells, called side population (SP), remains unlabelled and may be isolated on this basis. This population (0.15 to 1.2% of the total population) has characteristics of CSCs, that is, the ability to form neurospheres when cultured in defined medium over a long term (self-renewal) while retaining the ability to differentiate into neurons and glial cells at each passage (multipotency) and the ability to trigger tumor formation after injection into immunocompromised mice [59, 60]. Several surface markers are currently used to identify CSCs. As cited before, CSCs are identified as $CD44^+$ $CD24^{-/low}$ in breast tumors [44] while,

in gliomas, they have been identified mostly on the basis of the expression of CD133 (or prominin 1) [45]. However, the CD133 marker has been questioned since it is a target gene of HIF-1, one of the main transcription factors of hypoxia [61], and its expression can be increased by chemical or genetic dysfunction of mitochondria [62]. Thus, at least for gliomas, the identification of other CSCs markers, such as nestin, an intermediate filament, a marker of neural stem cells [63], or CD15 (SSEA1 or Lewis X) present in primary neurospheres [64], is under evaluation. Finally, if different surface markers have been described for CSCs from various tumors, most of these markers are shared with normal stem cells.

Normal mouse embryonic stem cells (ESCs) exhibit a bivalent metabolism (glycolytic or phosphorylative depending on the cell requirements). However, human ESCs exhibit a glycolytic metabolism, probably due to defective mitochondria [65]. Based on these studies or those in the early stage of embryos [66, 67], highly undifferentiated cells such as CSCs should be able to revert between aerobic glycolysis and glutaminolysis. Several studies have shown a glycolytic phenotype in CSCs with an overexpression of most glycolytic enzymes (Figure 1) [68, 69]. Several isoforms of LDH, known to be upregulated under hypoxia as well as c-MYC, are commonly highly expressed in CSCs, which facilitate the diversion of glucose carbons away from oxidative metabolism. However, in a similar way that some cancer cells exhibit an oxidative rather than glycolytic metabolism, CSCs have different metabolic profiles depending on their tissue of origin and their degree of differentiation. For example, highly undifferentiated liver cancers tend to be more glycolytic than tumor cells that retain some differentiation characteristics [70]. A recent study using a glioma stem cells model showed that these cells consumed less glucose and produced less lactate compared to their cancer cell counterparts [71].

Serine and glycine are both nonessential amino acids that can be taken up by cells or synthesized from 3-phosphoglycerate. These amino acids are important precursors for nucleotide and glutathione synthesis. In CSCs isolated from nonsmall cell lung cancer, Zhang et al. showed a high upregulation of genes involved in serine and glycine metabolism concomitant with an upregulation of glycolytic genes [72]. In particular, the expression of the glycine decarboxylase (GLDC) was markedly upregulated in these CSCs. These authors also showed that GLDC overexpression alone was able to transform NIH 3T3 cells *in vitro* and drive tumor formation *in vivo*, while silencing of this enzyme-diminished tumorigenicity.

In addition to the intrinsic needs of the cells, exogenous factors influence both cellular fate and metabolic processes. The resident microenvironment, also known as the niche, is an indispensable factor that distinguishes normal stem cells from CSCs. The niche is the source of molecules that activate or inhibit signal transduction pathways. While the stem cell microenvironment of a normal tissue is known to maintain a balance between self-renewal and differentiation [73–75], the tumor microenvironment required for the maintenance of CSCs is altered, retaining predominantly proproliferating signal [76, 77]. The role of the tumor microenvironment in tumor initiation and progression through stromal cells or immune cells, as well as alterations in extracellular modeling or oxygen concentration, is widely accepted [78]. These niches are characterized by a low oxygen concentration and as such promote a glycolytic phenotype mediated, in part, through the HIF-signaling pathway. During cancer initiation, a hypoxic environment would favor the activation of genes associated with "stemness" such as Notch or Oct4 as well as genes associated with the glycolytic switch, for example glucose transporters, (hexokinase) HK, PKM2, LDH, and PDK [79]. In fact, increased expression of nestin through the activation of the Notch-signaling pathway has been detected in glioma cell lines [80]. The capacity of CSCs to modulate the tumor microenvironment has also been suggested. In solid tumors, the adaptation of CSCs hypoxia resulting in a glycolytic shift would mediate the acidification of the tumor microenvironment. In fact, local pH measurements revealed a shift from 7.1 in normal brain tissue to 6.8 in brain tumors with some being as low as 5.9 [81]. This acidification promotes the maintenance of the stem cell phenotype. In addition, microenvironment acidification would in turn alter the activity of proteases that are implicated in the degradation of the extracellular matrix. In fact, several studies have shown that hypoxia promotes metastasis through HIF-dependent pathways [82] and through the activation of enzymes involved in the rigidity of the extracellular matrix such as lysyl oxidases [83]. On the clinical level, there is a direct correlation between the presence of a hypoxic core within the tumor and a poor prognosis for patients [82, 84, 85].

5. Resistance to Therapy of Cancer Cells

Evasion of programmed cell death or apoptosis has been recognized as one of the main alterations that dictate malignant growth and is a hallmark of most types of cancer [86]. Apoptosis can be triggered either by the intrinsic (mitochondrial) pathway or the extrinsic (death receptor) pathway. The central players in both pathways are the family of caspases (Figure 2). The activation of the intrinsic pathway induces mitochondrial membrane permeabilization leading to the release of apoptogenic proteins including cytochrome c, and ultimately to the activation of caspase cascade, DNA fragmentation, and cell death. The BCL-2 family of proteins, consisting of antiapoptotic, proapoptotic, and BH3-only proteins, plays a central role in controlling the intrinsic pathway. These proteins are located or translocated to the mitochondrial membrane and modulate apoptosis by altering the outer mitochondrial membrane permeability [87]. The extrinsic pathway is activated through the (tumor necrosis factor) TNF receptors. After binding of its ligand (TNFα, FAS-L, or TRAIL), the receptor oligomerizes, leading to the formation of the (death-inducing signaling complex) DISC (death-inducing signaling complex) with the recruitment of a specific adaptor protein leading ultimately to the activation of caspase 8. DISC activation will either directly activate effector caspases or cleave the BH3-only protein Bid, which in turn would engage the mitochondrial pathway through the activation of proapoptotic Bax. Apoptosis is also controlled by the Inhibitors of Apoptosis Proteins (IAPs), including

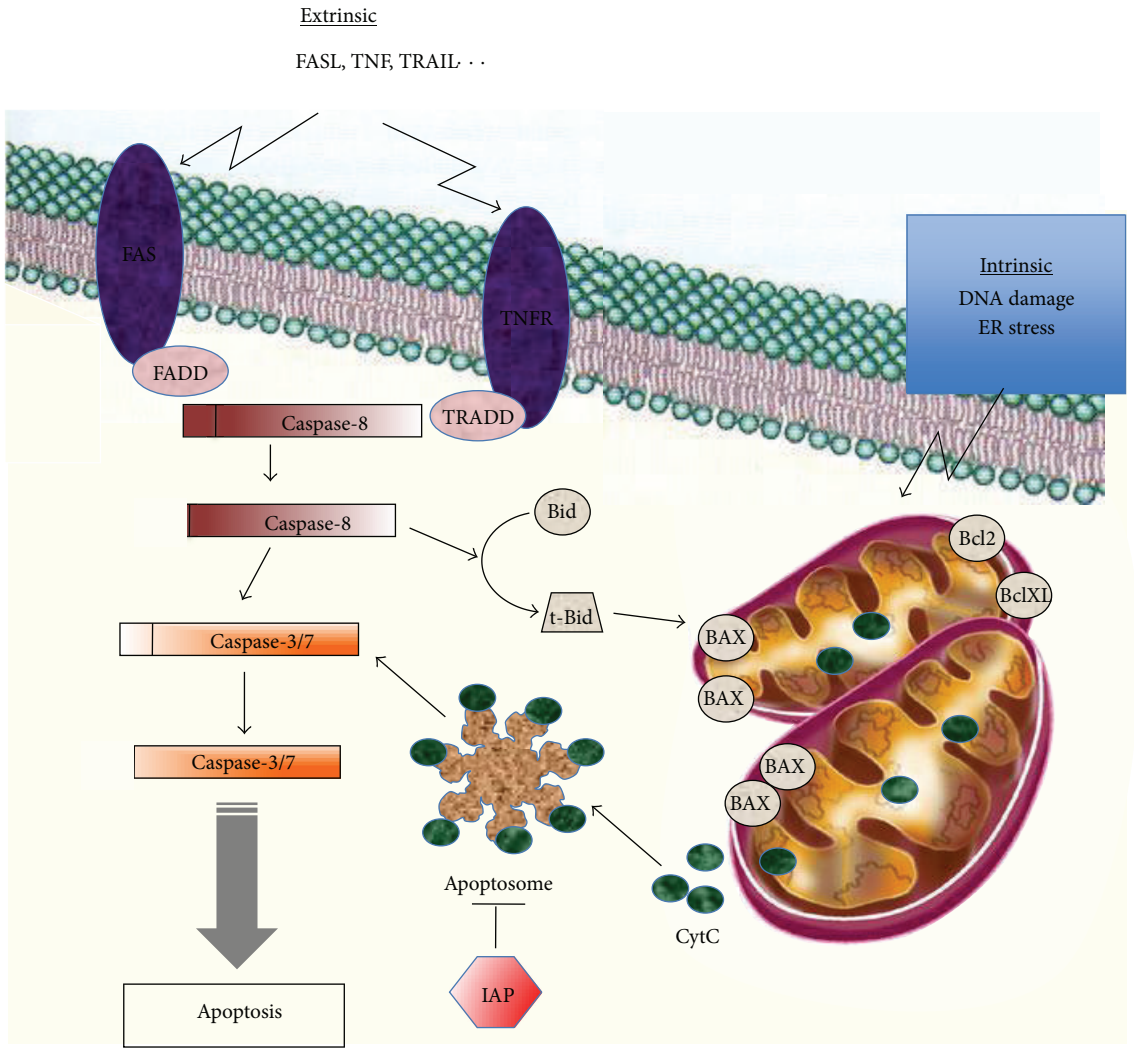

Figure 2: Intrinsic and extrinsic apoptotic pathways. Apoptosis can be triggered by the intrinsic mitochondrial pathway or through the extrinsic pathway involving the death receptors. The intrinsic apoptotic pathway is activated in response to various stimuli such as DNA damage, endoplasmic reticulum (ER) stress, or hypoxia. This pathway is mainly modulated through differential interactions between the antiapoptotic (Bcl-2, Bcl-XL), the proapoptotic (Bax, Bak), and the BH3-only proteins (Bad, Bid, Bim...). Bax, Bid, and Bim are initially inactive and must translocate to the mitochondria to induce apoptosis, either by binding via BH3 domains to Bcl-2, Bcl-XL and antagonizing their antiapoptotic functions or through the permeabilization of the mitochondrial membrane. Permeabilization of the mitochondrial membrane releases apoptogenic proteins, among which the cytochrome c is leading to the formation of the apoptosome, activation of caspase 9, and ultimately to the activation of effector caspases. In the extrinsic pathway, ligands (TNF, FASL, or TRAIL) bind to their specific death receptors, which lead to their oligomerization, recruitment of procaspase 8, and a specific adaptor protein (FADD and TRADD). The formation of the DISC induces autocatalysis of procaspase 8 into its active form, which in turn leads to the activation of the effector caspases.

survivin, and the FLIP proteins that inhibit the activation of caspase-8.

Antiapoptotic pathways are generally enhanced in tumor cells, which promote their survival but also render the cells more dependent on antiapoptotic pathways thus providing a potential therapeutic window. The intrinsic pathway of apoptosis is activated in the presence of most anticancer drugs and other stresses such as growth factor deprivation or DNA damage [75, 76]. It has been demonstrated that resistance to chemotherapy-induced apoptosis in several tumor cell types is controlled by antiapoptotic proteins Bcl-2, or Bcl-X_L while sensitivity to apoptosis *in vivo* is associated with increased levels of Bax [88]. Recently, it has been shown that Bcl-X_L protects against apoptosis through a mechanism independent of proapoptotic proteins Bax/Bak, by reducing glucose-derived citrate, which in turn caused a decrease in the levels of acetyl CoA and protein N-alpha acetylation [89]. The latter would affect protein activity, stability, assembly, and localization within the cell.

The activation of the extrinsic pathway promotes apoptosis in many types of tumors. However, toxic side-effects were observed with recombinant TNF and agonistic anti-FAS

antibodies limiting their therapeutic use [90]. A potentially more promising approach involves targeting the TRAIL receptors. Phase I clinical trials have established the safety and tolerability of these TRAIL agonists in patients [91]. Phase II trials are currently evaluating the therapeutic efficacy of TRAIL agonists as single agents or in combination with established cancer therapy. Unfortunately, about 50% of cancer cell lines are resistant towards TRAIL-induced apoptosis [92] and furthermore, TRAIL receptors can elicit prosurvival or proinvasive effects, both of which are counterproductive in treatment [93]. Finally, some recent studies have shown that cell death resistance could be linked to alterations in the structure of mitochondria [83, 84].

6. Mechanism of Cell Death Resistance in CSCs

Cancer stem cells being more quiescent are more resistant to apoptosis. The role of Bcl-2 in protecting hematopoietic CSCs against apoptosis has been demonstrated both *in vitro* and *in vivo* [94] as well as in response to radiation [95]. Similarly, $Bax^{-/-}$ mice exhibit increased multipotent progenitor cells [96]. In gliomas, antiapoptotic genes, including *flip*, *bcl-2,* and *bcl-xl*, as well as IAP family members (*xiap, ciap1, ciap2, naip,* and *survivin*) are found at higher levels in CSCs ($CD133^+$), and this correlates with enhanced drug resistance to different agents including temozolomide, carboplatin, VP16, and Taxol [88]. Furthermore, a high Mcl-1 expression was associated with resistance to ABT-737, a BH3-mimetic, in glioma stem cells [97].

Resistance to cell death upon radio- or chemotherapy is also mediated through the DNA damage repair (DDR) machinery. In fact, glioma CSCs exhibit a higher capacity of DDR mediated, to some extent, through an elevated activation of checkpoint kinases Chk1 and Chk2, in response to radiation [98]. Furthermore, the DNA repair enzyme MGMT (O6-methylguanine-methyltransferase) is usually overexpressed in these cells [88]. In fact, several studies have shown that MGMT overexpression predicts a patient response to temozolomide, an alkylating drug that prolongs survival when administered during and after radiotherapy in first-line treatment in GBM [99]. Similar studies in mesenchymal stem cells (MSCs) have highlighted the tumor-promoting role of p21 [100]. The transformation of these cells has been to some extent associated with deregulation of the cell-cycle proteins p16 and CDK triggering an increase in resistance to apoptosis [101]. The activation of DDR through p21 appears to be implicated in leukemia CSCs self-renewal [102]. Interestingly, human MSCs are resistant to apoptosis when undifferentiated but become sensitive to cell death upon the initiation of differentiation [103].

Glioma CSCs are also resistant to TRAIL-induced apoptosis partially through methylation of caspase 8 [104] although it was suggested that this resistance could be overcome by treating with the proteasome inhibitor bortezomib [105] or with cisplatin in breast CSCs [106]. Sensitivity to TRAIL-induced apoptosis is increased in colon CSCs, defined as the SP by Hoechst 33342 staining. These cells, known to be resistant to chemotherapy, express higher levels of TRAIL-receptor 1 (TRAIL-R1) that correlates with increased sensitivity to TRAIL-induced apoptosis [107].

Finally, the efficiency of apoptosis targeting agents is limited by the presence in CSCs of active transmembrane ATP-binding cassette (ABC) transporters involved in the efflux of drugs. For example, enhanced resistance of glioma CSCs ($CD133^+$) to temozolomide or etoposide is mediated by a higher expression of ABCG2 [88]. Breast cancer cells and isolated mammary gland CSCs are also less sensitive to treatment through increased activity of Wnt pathway leading to an overexpression of MDR1 [108]. Current hypotheses suggest that this CSCs resistance to treatment could also be mediated through the protection accorded by the niche.

7. Impact of Metabolism on Cell Death

We have illustrated above an increasing amount of evidence suggesting that metabolic alterations are primary events in the transformation process, whether this is through activation of oncogenes, inactivation of tumor suppressors, or mutations in genes encoding metabolic enzymes. However, how specific metabolites contribute to apoptosis in tumor cells remains a central question. One possible link between metabolic change and resistance to apoptosis is the association of HKs with the voltage-dependent channel protein (VDAC) under glycolytic metabolism. While this interaction facilitates the phosphorylation of glucose using ATP generated by mitochondria, it also prevents the binding of proapoptotic proteins such as Bak with VDAC, thereby preventing apoptosis [96, 97]. Another glycolytic enzyme with a proapoptotic function is Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH), which translocates to the nucleus in cultured neurons and induces neuronal death when over-expressed [109]. The fact that GAPDH is over-expressed in cancer cells seems paradoxical since it was shown that GAPDH is able to prevent caspase-independent cell death by increasing the amount of intracellular ATP and by stimulating autophagy [99, 100].

Tp53-Induced Glycolysis and Apoptosis Regulator (TIGAR), a target of p53, inhibits glycolysis by reducing the level of FBP. Glucose is then redirected into the pentose phosphate pathway (PPP) to produce NADH and nucleotides instigating an increase in glutathione. As such, TIGAR decreases the sensitivity of cells to p53 and other apoptotic signals associated with ROS [101, 102]. Similarly, an over-expression of PFK diverts glucose from glycolysis to the PPP and increases the resistance to oxidative stress [110].

Attempts have been made to modulate metabolic reprogramming by treating with compounds that inhibit glycolysis. Several studies show that glucose deprivation leads to cell death. For example, glioma cells cultured in the absence of glucose die by ROS-induced apoptosis suggesting that, in the absence of glucose, these cells are able to change their metabolism and use their mitochondria to produce ATP [111]. Another study showed that a shortage of glucose would induce cell death in cells deficient in Bax and Bak, effectors of mitochondrial permeabilization via an unconventional pathway requiring caspase 8 [112]. This effect is highlighted

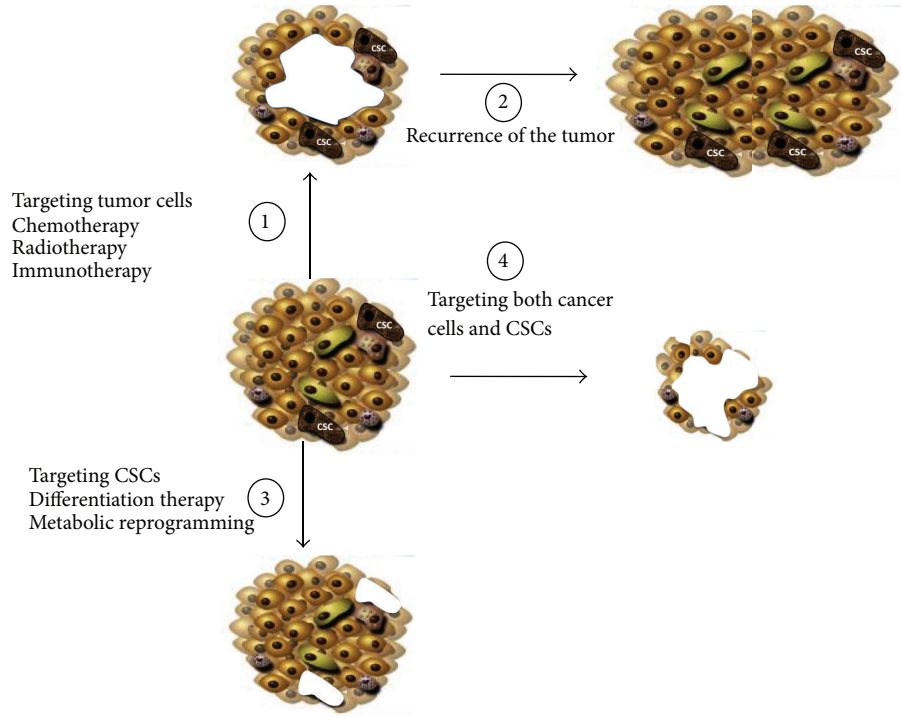

Figure 3: Needs of combinatorial therapies that target cancer cells and CSCs. A tumor is a complex mix of cancer cells including differentiated cells at different stages as well as CSCs. Current treatments kill cancer cells without affecting cancer stem cells (1). However, since CSCs are not affected, there is a major risk of tumor recurrence (2). Targeting only CSCs might result in a reduced number of matured cells but that will not be sufficient to eradicate the tumor (3). Thus, cancer therapy should ideally target both CSCs and matured cells (4) by generally increasing sensitivity to cell death of all the cell types as well as reducing the proliferation of matured cells and inducing differentiation and sensibility to cell death of CSCs.

using the glucose analog, 2-deoxyglucose (2-DG), which accumulates in cells and inhibits HK. At high concentrations, 2-DG causes a decrease in ATP levels resulting in cell death, especially in cells with mitochondrial defects or under hypoxia [113]. This compound has entered numerous clinical trials in combination with other agents and seems to potentiate the effect of radiotherapy, at least in patients with brain tumors [114]. Dichloroacetate (DCA) is another molecule involved in the downregulation of glycolysis. This small molecule inhibits mitochondrial PDK, forcing pyruvate into the mitochondria thereby increasing mitochondrial metabolism [115]. Indeed, DCA decreases tumor growth *in vitro* and *in vivo* without affecting normal tissue [116–118]. While DCA alone has no effect on apoptosis in glioma CSCs, it induces a Bax-dependent apoptosis in these cells when combined with etoposide or radiation [69]. Recently, it was shown that DCA, already used in the clinical treatment of genetic mitochondrial diseases [119], could be used in patients with GBM by inhibiting PDK over-expressed in these tumors [120]. One of the main properties of this molecule is its blood-brain barrier permeability. Furthermore, a direct consequence of downregulating glycolysis by DCA is an increase in intracellular pH, which in turn decreases the invasive ability of tumors.

PKM2 is a promising target for potential therapeutic approaches since the ratio of PKM2 tetramer: dimer has severe consequences on metabolism, proliferation and the tumorigenic capacity of the cells [121, 122]. Furthermore, this isoform can translocate into the nucleus where it can either induce cell death upon various apoptotic stimuli such as UV or H_2O_2 [123] or interact with transcription factors involved in the "stemness" such as Oct4 [121, 122]. Many inhibitors capable of blocking the allosteric regulation of the M2 isoform are currently under investigation. It was shown that a peptide (Aptamer 9) blocked PKM2 in its inactive conformation thereby decreasing cell proliferation even in the presence of high concentrations of glucose [124]. A recent study revealed new activators (diarylsulfonamides) of this enzyme, the effects of which are still to be demonstrated [125].

Differentiation therapy has also been exploited with Bone Morphogenetic Protein-4 (BMP4) treatment to induce glial differentiation reducing tumor growth in gliomas. Interestingly, after this treatment, glioma CSCs are unable to form tumors after transplantation in series in immunocompromised animals [126]. This study suggests a new treatment for GBM that would force the CSCs to enter differentiation, resulting in, firstly, a reduction in tumor mass and, secondly, a decrease in resistance to apoptosis of these cells.

8. Conclusion

The successful elimination of a cancer requires an anticancer therapy that will affect both differentiated cancer cells and CSCs (Figure 3). At present, conventional therapy that

includes radio-, chemo-, and immunotherapy kills rapidly proliferating and differentiated cells. These treatments may cause the tumor to shrink but will not prevent tumor recurrence. Thus, a combination of treatments targeting both rapidly proliferating cancer cells and quiescent or slow-proliferating CSCs would be ideal. Therefore, it is essential to identify specific markers that distinguish between tumorigenic and nontumorigenic stem cells. These therapeutic strategies for CSCs include targeting pathways involved in the self-renewal process, differentiation, and "exit" from the niche. Furthermore, a reversal of tumor metabolism to "normal" might impair tumor growth of cancer cells, causing tumor regression, and differentiation/sensitization to cell death of CSCs, impairing the recurrence of the tumor.

Abbreviations

Chk:	Checkpoint kinase
DCA:	DiChloroAcetate
2-DG:	2-DeoxyGlucose
αKG:	α-KetoGlutarate
CSCs:	Cancer stem cells
DISC:	Death-inducing signaling complex;
DDR:	DNA damage repair
FBP:	Fructose-1,6-biPhosphate
FH:	Fumarate hydratase
GBM:	Glioblastoma multiforme
GLS:	Glutamine synthase
GLUT:	Glucose transporters
2-HG:	2D-hydroxyGlutarate
HK:	Hexokinase
HIF:	Hypoxia inducible factor
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
IDH:	Isocitrate dehydrogenase
LDH:	Lactate dehydrogenase
MGMT:	O6-methylguanine-methyltransferase
MSCs:	Mesenchymal stem cells
OAA:	Oxaloacetate
OXPHOS:	Oxidative phosphorylation
PPP:	Pentose phosphate pathway
PI3K:	Phospho-inositol 3 kinase (PI3K)
PHD-2:	Prolyl hydroxylase domain protein-2
PDH:	Pyruvate dehydrogenase
PDK:	PDH-kinase;
PK:	Pyruvate kinase
SDH:	Succinate dehydrogenase
TCA:	Tricarboxylic acid cycle.

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Review Article

Alternative Cell Death Pathways and Cell Metabolism

Simone Fulda

Institute for Experimental Cancer Research in Pediatrics, Goethe-University Frankfurt, Komturstraße 3a, 60528 Frankfurt, Germany

Correspondence should be addressed to Simone Fulda; simone.fulda@kgu.de

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While necroptosis has for long been viewed as an accidental mode of cell death triggered by physical or chemical damage, it has become clear over the last years that necroptosis can also represent a programmed form of cell death in mammalian cells. Key discoveries in the field of cell death research, including the identification of critical components of the necroptotic machinery, led to a revised concept of cell death signaling programs. Several regulatory check and balances are in place in order to ensure that necroptosis is tightly controlled according to environmental cues and cellular needs. This network of regulatory mechanisms includes metabolic pathways, especially those linked to mitochondrial signaling events. A better understanding of these signal transduction mechanisms will likely contribute to open new avenues to exploit our knowledge on the regulation of necroptosis signaling for therapeutic application in the treatment of human diseases.

1. Introduction

There are several forms of cell death in mammalian cells, among them apoptosis and necrosis as the two best characterized and most intensively studied modes of cell death [1]. Apoptosis is characterized by a series of programmed events, including membrane blebbing, caspase activation, and internucleosomal DNA fragmentation [1]. In contrast to apoptosis, necrosis represents a form of cell death that typically lacks the activation of caspases, while it involves swelling of mitochondria, irreversible damage to cellular membranes, eventually leading to spilling of the intracellular content into the surrounding environment [1]. In addition, a regulated form of necrosis, that is, necroptosis, has recently been identified that proceeds in a programmed and controlled manner [2]. Necroptosis refers to RIP1- and/or RIP3-dependent regulated necrosis [1]. A better understanding of the molecular mechanisms that regulate necroptosis signal transduction may open new perspectives for targeted modulation and therapeutic exploitation of necroptosis signaling. This paper will focus on the crosstalk between necroptosis and metabolic signaling events, in particular redox signaling.

2. Necroptosis Signaling

There are various stimuli that can engage necroptosis, including ligands of the death receptor family such as TNFα, chemical or physical damage, or hypoxic conditions [2]. Since necroptosis represents a programmed form of cell death, there are clearly delineated signal transduction cascades that eventually lead to the demise of the cell.

For example, in TNFα-triggered necroptosis, binding of the death receptor ligand TNFα to cognate plasma membrane receptors on the cell surface, that is, TNF receptor 1 (TNFR1) as the main receptor for TNFα, engages an intracellular signaling machinery [3]. This involves the recruitment of adaptor molecules such as TRADD signaling molecules such as RIP1, cIAP1, cIAP2, TRAF2, and TRAF5 to the cytoplasmic part of TNFR1. The recruitment of the signaling molecules leads to the formation of the so-called complex I, a multimeric protein complex that leads to the polyubiquitination of RIP1 by cIAP proteins. Following its polyubiquitination, RIP1 can serve as docking site for TAK1, TAB2 and TAB3 which results in the activation of the transcription factor NF-κB. Upon binding of TNFα, TNFR1 is internalized in order to terminate signal transduction via the ligated

membrane-bound receptor which subsequently leads to a change in the molecular composition of the interacting signaling proteins. This enables the formation of the so-called TNFR1 complex II which contains core components such as FADD, RIP1, and caspase-8 and serves as a platform for caspase-8 activation and initiation of apoptosis. However, if caspase-8 is insufficiently activated in this complex, an alternative complex can be formed, that is, the so-called necrosome complex [2]. This necrosome complex contains FADD, RIP, and in addition also RIP3 and is critically required for the initiation of necroptotic cell death. Since RIP1 can be a part of cell death and survival signaling complexes, posttranslational modifications of RIP1 ensure that fine tuning of RIP1 functions in apoptosis versus necroptosis versus NF-κB signaling is achieved. Accordingly, the ubiquitination of RIP1 favors the formation of the cell death-initiating platforms, whereas ubiquitinated RIP is a component of the NF-κB-signaling cascade [2]. Upon the formation of the necrosome complex, RIP1 and RIP3 become phosphorylated and activated which in turn engages downstream signaling events that eventually lead to necroptotic cell death [4]. Of note, these signaling networks also involve several crosstalks with cellular metabolic pathways.

3. Regulation of Necroptosis by Redox Signaling

The regulatory mechanisms that are involved in the control of the initiation and propagation of necroptosis are still largely unknown. There is accumulating evidence that redox processes play an important role in the regulation of necroptosis. For example, the production of ROS is rapidly increased during the early stages of necroptosis [5, 6] indicating that ROS generation may be involved in mediating necroptosis. Indeed, it has been shown that the inhibition of the production of reactive oxygen species (ROS) also reduces the induction of necroptosis [5, 7].

What are the generator systems for ROS production during necroptosis? There are several potential intracellular sites that can contribute to ROS production in the course of necroptosis. In principle, they can be divided into mitochondrial generator systems and extramitochondrial sites for ROS production. Within mitochondria, complexes I and III of the respiratory chain are considered as main sites for ROS production during programmed cell death [8]. In addition, the mitochondrial adenine-nucleotide translocates (ANT) can contribute to ROS production in the course of cell death. ANT is localized at the inner mitochondrial membrane and responsible for the exchange of ADP against ATP [9]. The inhibition of ANT leads to a decrease of ADP levels with concomitant increase in ATP in the mitochondrial matrix, which in turn reduces the activity of the ATP synthase and hyperpolarization of the mitochondrial membrane potential, thereby favoring the production of ROS [10]. Since RIP1 has been described to negatively regulate ANT activity, it is tempting to speculate that elevated RIP1 activity during necroptosis may inactivate ANT, thereby favoring the production of ROS species.

As far as extramitochondrial sources of ROS are concerned, NOX NADPH oxidases represent the main sources of ROS production in the extramitochondrial compartment [11]. It is interesting to note that the prototypic necroptosis stimulus TNFα has recently been reported to increase the activity of NOX1 complex via a mechanism involving RIP1 [12]. TNFR1 complex I has also been reported to serve as platform, which enables the docking of the NADPH oxidase NOX1 at the plasma membrane, thereby promoting the generation of ROS [12]. This involves the TNFα-stimulated association of NADPH oxidase organizer 1 (NOXO1) subunit with RIP1, TRADD and riboflavin kinase, which in turn results in ROS production via NOX1 [12].

4. ROS Species as Executioners of Necroptosis

Already twenty years ago the execution of necrotic cell death has been associated with ROS species as critical mediators of cell death. It was reported that complex I-mediated production of ROS species is required for necrotic cell death in response to TNFα stimulation [8]. In addition, ROS production within the mitochondria has been associated with structural changes and damage to organelles such as mitochondria and the endoplasmic reticulum [8, 13]. In addition to mitochondrial ROS, the generation of ROS from extramitochondrial sources, for example, via the plasma membrane-associated NADPH oxidase NOX1, has been shown to mediate necrotic cell death upon stimulation with TNFα [12]. ROS generation by NOX1 might not only result in lipid peroxidation and membrane damage but may also engage a feed-forward amplification loop to trigger further ROS production via the mitochondrial respiratory chain. Another amplification loop might involve the lysosomal compartment where hydrogen peroxide can interact with ferrous ions to produce hydroxyl radical (Fenton reaction), a highly reactive ROS species [14]. Such amplification loops can lead to the overproduction of ROS, for example, at the mitochondrial respiratory chain. This bears the danger of a lethal vicious cycle eventually resulting in the generation of reactive nitrogen species (RNS). RNS species can function as oxidants to produce protein or lipid oxidation, thereby altering protein function and causing membrane damage [15].

Moreover, ROS generation has also been linked to mitochondrial bioenergetics. To this end, advanced glycation end products (AGE) which are generated as the result of several chemical reactions in response to elevated levels of extracellular glucose have been reported to bind to receptors on the cell surface and to promote ROS production [16].

5. Bioenergetic Regulation of Necroptosis

Apoptosis and necrosis not only represent morphologically two distinct forms of cell death but also from the aspect of bioenergetics demands. The initial characterization of apoptotic and necrotic cell death has revealed that intracellular adenosine triphosphate (ATP) content represents a central regulator in the decision on the mode of cell death, that is, apoptosis and necrosis. Accordingly, human T-cells have

been reported to switch from apoptosis in response to CD95 stimulation or treatment with staurosporine towards necrosis upon depletion of ATP [17]. In this model, the generation of ATP by either the mitochondrial respiratory chain or by glycolysis was shown to be necessary to provide the energetic supply to execute apoptosis via DNA fragmentation [17]. The addition of extramitochondrial ATP, for example, by repletion of glucose, resulted in restored ability of T cells to undergo apoptotic cell death [17]. Subsequently, different steps in the apoptotic signaling cascade were shown to depend on sufficient supply of bioenergetics substrates and ATP consumption, for example, activity of the translational machinery, protein degradation via the ubiquitin proteasome system, and activity of DNA repair enzymes such as PARP1 [18–20]. PARP1 has been described to play an important role in the metabolic regulation of cell death. PARP1 is localized in the nucleus and can sense DNA damage, which leads to the overactivation of PARP1 when DNA damage is extensive [21, 22]. PARP overactivation then causes depletion of NAD and ATP and an acute bioenergetic depletion, which promotes the release of apoptosis-inducing factor (AIF) from the interspace of mitochondria and its translocation to the nuclear compartment [23]. Within the nucleus, AIF is supposed to be required for large-scale DNA fragmentation in a caspase-independent manner [23]. This type of PARP1-dependent necrotic cell death has been implicated in several pathophysiological conditions, for example, upon cerebral ischemia, oxygen-glucose depletion, or exposure to alkylating DNA-damaging agents [21, 22, 24].

The mitochondrial permeability transition pore complex (PTPC) represents another multimeric protein complex that has been implied in the bioenergetic control of several cell death modalities [25]. The PTPC is composed of ANT, an integral protein of the inner mitochondrial membrane that is responsible for the exchange of ATP with ADP across mitochondrial membranes [9], voltage-dependent anion channel (VDAC), a protein of the outer mitochondrial membrane [26], and cyclophilin D, which is localized to the mitochondrial matrix [27]. The activity of VDAC is tightly controlled by various mechanisms including proteins of the Bcl-2 family, ions, and metabolites. The key role of VDAC in the regulation of necrotic cell death is underlined by genetic evidence showing that mice deficient in cyclophilin D are protected against ischemic injury [22, 28].

Downstream of the RIP1/RIP3 necrosome complex, metabolic pathways including glycogenolysis, and glutaminolysis have also been implicated to mediate signaling events during necroptosis. RIP3 has been shown to promote the activity of several metabolic enzymes including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), and glutamate dehydrogenase 1 (GLUD1) [6]. PYGL is a key enzyme in the catabolic metabolism of glycogen causing the conversion of glycogen into glucose-1-phosphate which in turn can be modified into glucose-6-phosphate, a substrate for glycolysis that can promote the generation of ROS via mitochondrial metabolic burst. In addition, RIP3 was shown to activate GLUL and GLUD1 [6], a cytosolic and mitochondrial enzyme, respectively, involved in glutaminolysis. Enhanced glutaminolysis can engage the Krebs cycle, eventually contributing to increased ROS generation. Thus, RIP3-dependent changes in glycogenolysis and glutaminolysis can result in enhanced energy metabolism and increased production of ROS.

6. Conclusions

Since its discovery, necroptosis has evolved as one of the key programmed cell death pathways in mammalian cells, which is deregulated in numerous human diseases. Thanks to enormous research efforts, considerable advances have been achieved in the identification of the signal transduction steps and control mechanisms that are involved in the control of necroptosis signaling pathways. Metabolic events including redox signaling represent critical events that are involved in the regulation of necroptosis signaling. Further insights into the fine-tuning of signaling processes will likely pave the avenue towards novel opportunities to target specific intervention points within in the necroptosis signaling cascade in order to achieve therapeutic benefits.

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