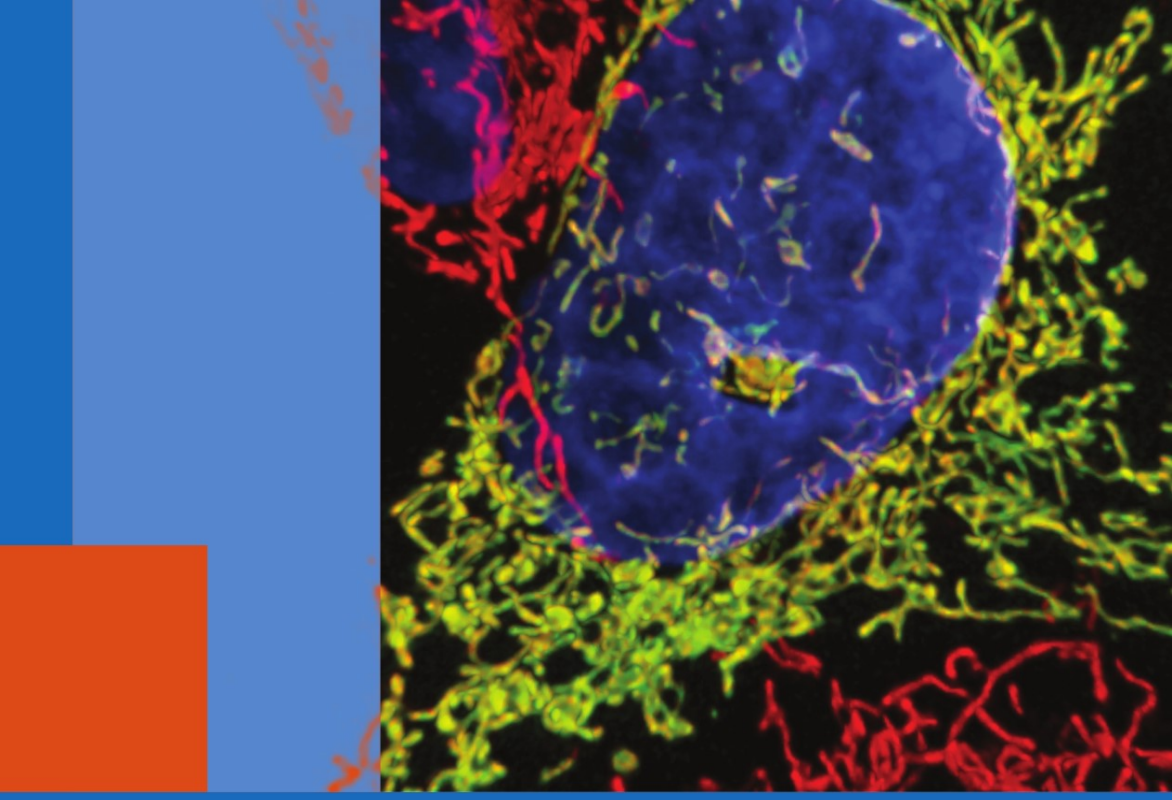

Mitochondria and Cancer

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Leslie C. Costello
Editors

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Preface

We are entering into an era of re-awakening of the importance and interest in the role of altered intermediary metabolism in the development and progression of malignancy. Therefore, this book *Mitochondria and Cancer* is timely and relevant to contemporary and future research, training, and education. The hallmark studies of Otto Warburg et al. (1926) sparked the era of tumor cell metabolism. From then until around 1980, studies of intermediary metabolism of normal and malignant cells were major areas of research and of graduate and postgraduate training in biomedical sciences. This period was dominated by mitochondrial studies and by those extraordinary outstanding scientists who were affectionately referred to as the "mitochondriacs". However, the advent, development, and subsequent dominance of molecular genetics/molecular biology and molecular technology in biomedical research were accompanied by the nearly complete submersion of interest in areas of intermediary metabolism and tumor cell metabolism. For the past 30 or so years, graduate and postgraduate training, experience and focus in biochemistry, metabolism, enzymology, cell physiology have declined, being displaced by the dominance of molecular technologies. The recent revelations and advances in molecular genetics/molecular biology and the development of molecular and nanotechnology provide new dimensions to apply to the understanding and elucidation of the role of intermediary metabolism and mitochondrial function in the development and progression of cancers. To address the issues of tumor metabolism, the present and future generations of researchers must integrate the knowledge and methods of biochemistry with the knowledge and technology of molecular biology.

The focus on mitochondria is central to discussions and issues of intermediary metabolism. The genetic transformation of normal functional cells to neoplastic cells imposes the need for alterations in intermediary metabolism and mitochondrial function to provide the bioenergetic/synthetic/growth requirements for the progression of the neoplastic cell to malignancy. In the absence of the metabolic transformation, the neoplastic malignant cell will not achieve its malignant potential. Thus, it is critical to establish the role of the mitochondria in the malignant process. Moreover, specific alterations in different cancer types must be identified. Such information will provide new approaches to the treatment and perhaps prevention of differing cancers. The advent of Metabolomics for the detection of

cancer and progression of malignancy is dependent upon the elucidation and identification of altered metabolism and mitochondrial function. Thus, this exciting journey in cancer research has barely begun and has a long way to go. This book, *Mitochondria and Cancer*, and its subject matter provide a roadmap and the preparation to proceed on this journey.

We are greatly indebted to the contributing authors for their enthusiastic support and writing a chapter in their area of expertise. We are grateful to Ms. Donna Ovak for secretarial help and our families for their patience and support in putting together this book. This book is dedicated to past, present, and future "mitochondriacs" around the world.

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Mitochondria and Cancer

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Introduction

Mitochondria have been an area of scientific study for more than 100 years (Table 1). It was in the early twentieth century that Otto Warburg first described differences in the mitochondria of tumor vs. normal cells. He observed that tumor cells had an increased rate of aerobic glycolysis compared with normal cells. He hypothesized that this increase was due to the impairment in the respiratory capacity of tumor cells (Warburg 1930, 1956). This was the first of several notable differences observed between the mitochondria of normal and transformed cells that were subsequently discovered (reviewed in Weinhouse 1955; Pedersen 1978; Carafoli 1980; Modica-Napolitano and Singh 2002, 2004; Modica-Napolitano et al. 2007). The physical structure, composition, and function of mitochondria differ greatly from that of tumor and normal cells. For example, the mitochondria of many rapidly growing tumors are fewer in number, smaller, and have fewer cristae than do mitochondria from slowly growing tumors, which tend to have characteristics more closely resembling those of normal cells. Polypeptide profiles, as well as lipid composition of the inner mitochondrial membrane of tumor cells, differ from those of normal cells. Additional differences between the mitochondria of normal vs. transformed cells also have been described with regard to the preference for substrates, mitochondrial membrane potential, rates of electron transfer, anion transport, protein synthesis, organelle turnover, and reactive oxygen species (ROS) production.

This chapter begins with a general overview of mitochondrial structure and function and then outlines more specifically the metabolic and genetic alterations in mitochondria associated with human cancers and their clinical implications.

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Table 1 Historical perspective on mitochondria

1890s	First description of mitochondria in cells as granules
	Altman speculated that these granules were basic units of cellular activity
	Benda first used the term 'mitochondrion', which means thread-like granule
1930s	Warburg described increased rates of glycolysis and mitochondrial defect in cancer cells
	Krebs formulated the urea and TCA cycle
1940s	Advances in cell fractionation techniques leads to the identification of mitochondria as centers of energy metabolism
	Succinate oxidase and cytochrome oxidase activities, as well as fatty acid oxidation and TCA cycle enzymes, were found in the mitochondria
1950s	Electron microscopy was used to identify mitochondria
	Cytoplasmic inheritance was described in yeast
	Mitochondria were shown to produce significant levels of hydrogen peroxide
	Respiratory chain analysis in the mitochondria was defined
1960s	Characterization of electron transport chain complexes
	Characterization of electron transfer molecules Coenzyme Q and cytochrome *c*
	Mitochondrial DNA was discovered
	Peter Mitchell described the chemiosmotic theory
1980s	All mitochondrial DNA genes were identified
	Complete sequence of human mitochondrial genome
	Mitochondrial diseases are first identified
1990s	Transformation of mtDNA in yeast by microprojectiles
	Role of mitochondria in apoptosis was first established
	Demonstration of complex V molecular rotation
	Crystal structure of complexes III, IV and F1 ATP synthase determined

Mitochondrial Structure

Mitochondrial structure varies from tissue to tissue. Mitochondria form a dynamic, filamentous network and are extending, contracting, fragmenting, and fusing with one another as they move in three dimensions. Distribution of mitochondria in the cytoplasm seems to be dependent on a microtubule-structured network (Aufderheide 1980; Summerhayes et al. 1983). Mitochondria are double-membrane organelles that are ~1–2 μm in length and 0.5–1 μm in width. The outer membrane encloses the entire contents of the mitochondrion. The inner membrane has a much greater surface area and forms a series of folds, or invaginations, called cristae. These invaginations allow for an increased surface area of the inner membrane, and thus, greater capacity for oxidative phosphorylation and energy production. The two membranes make two distinct compartments within the mitochondrion. The intermembrane space is located between the outer and inner membranes and the matrix is the space enclosed by the inner mitochondrial membrane. The transport of molecules and proteins into and out of these compartments is highly regulated by specific channels and shuttles.

Mitochondrial Function

In general, mitochondrial function varies from tissue to tissue. Mitochondria play a central role in oxidative metabolism in which carbohydrates and fatty acids are catabolized into energy equivalents (reviewed in Tzagoloff 1982; Gibson 2005). Carbohydrate catabolism begins with the import of pyruvate into the mitochondria, which is subsequently decarboxylated to acetyl CoA. Acetyl CoA then enters the tricarboxylic acid (TCA) cycle and undergoes an eight-step catabolic process, which results in the production of three molecules of reduced nicotinamide adenine dinucleotide (NADH), one molecule of reduced flavin adenine dinucleotide ($FADH_2$), one molecule of GTP (the energetic equivalent of ATP), and two molecules of CO_2 per cycle (Fig. 1).

The ETC is a series of protein complexes that takes electrons from reduced NADH and succinate in the TCA cycle and passes them to O_2 to form H_2O; in the process, protons (H^+) are translocated from the mitochondrial matrix to the intermembrane space. The chemiosmotic theory, proposed by Mitchell in 1961 (and for which he received the Nobel Prize in Chemistry in 1978), explains how the H^+ gradient across the inner mitochondrial membrane created by complex I, III, and IV governs the production of ATP at complex V (Mitchell 1961). Electrons enter the ETC through complex I (NADH dehydrogenase) or through complex II (succinate dehydrogenase, SDH) by the dehydrogenation of TCA cycle intermediates. The electrons are then transferred to Coenzyme Q (CoQ) and are cycled in the Q cycle

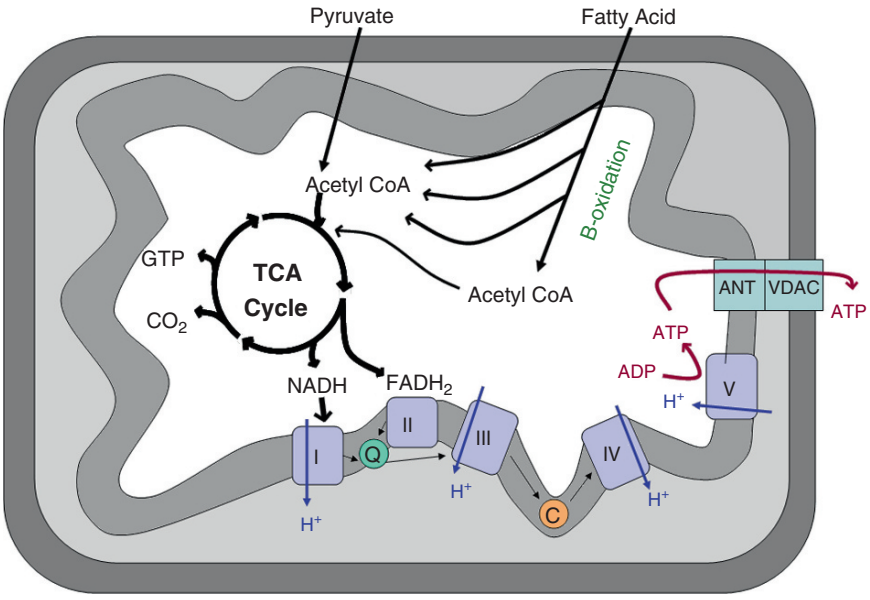

Fig. 1 Schematic of mitochondrial metabolism. See text for details

within complex III, resulting in the reduction of cytochrome *c*. Then, cytochrome *c* transfers the electrons to complex IV (cytochrome *c* oxidase), in which O_2 is the terminal electron acceptor and undergoes a four-electron reduction to H_2O (Voet et al. 2002). In this process, H^+ are pumped into the intermembrane space by complexes I, III, and IV. The translocation of H^+ from an area of low $[H^+]$ to high $[H^+]$ by the ETC complexes creates a proton motive force that drives ATP synthesis by complex V (Fig. 1) (Nelson and Cox 2000). The ATP produced is either used in the mitochondria or transported across the inner and outer mitochondrial membranes by the adenine nucleotide translocase (ANT) and voltage-dependent anion channel (VDAC), respectively.

Fatty acid oxidation is another important metabolic pathway that takes place in the mitochondria (Fig. 1). In each cycle of fatty acid oxidation, a fatty acid molecule is oxidatively decarboxylated into one molecule of acetyl CoA and one molecule of acyl CoA that is two carbons shorter than the starting fatty acid. This cycle continues until the original fatty acid molecule is decomposed entirely to acetyl CoA (e.g., the 16-carbon palmitoyl CoA would undergo seven rounds of beta-oxidation to yield eight molecules of acetyl CoA). The acetyl CoA produced in each round of the beta-oxidation pathway is then able to enter the TCA cycle.

In addition to the metabolism of carbohydrates and fatty acids, mitochondria are involved in gluconeogenesis and the urea cycle as well as the regeneration of cytosolic NAD^+ (required for the substrate-level phosphorylation in glycolysis) and in the intracellular homeostasis of inorganic ions such as calcium and phosphate. Mitochondria also play an important role in cell signaling events that lead to apoptosis, gene regulation, or cell cycle control (Petit and Kroemer 1998; Zamzami et al. 1996; Kroemer et al. 2007).

Mitochondrial Genome

The number of mitochondria and mitochondrial DNA (mtDNA) varies from tissue to tissue. The human mitochondrial genome is a 16.6-kb circular DNA that encodes for 13 of the proteins in the ETC and the displacement loop (D-loop), as well as 2 rRNAs and 22 tRNAs necessary for the translation of mitochondrial genes (Fig. 2a) (Bianchi et al. 2001; Singh 1998; Taanman 1999). The mitochondrially encoded proteins include seven subunits of oxidative phosphorylation complex I, one subunit of complex III, three subunits of complex IV, and two subunits of complex V (Fig. 2b). The D-loop is a triple-stranded noncoding region of mtDNA (np 16024–516) that contains *cis*-regulatory elements required for the replication and transcription of the mtDNA. All other mitochondrial proteins, including those involved in the replication, transcription and translation of mtDNA, are nuclear encoded. These nuclear-encoded proteins are imported by specialized protein complexes on the inner and outer mitochondrial membrane (Schatz 1996).

Although mtDNA represents less than 1% of total cellular DNA, its gene products are essential for normal cellular function. Mammalian mtDNA contains

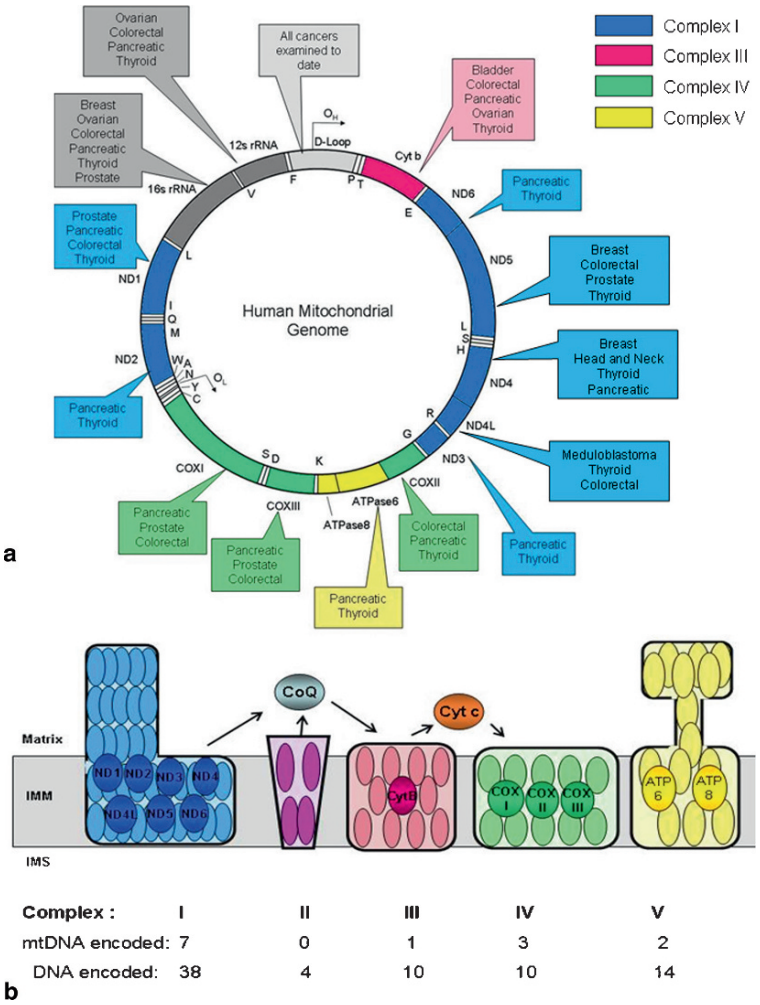

Fig. 2 Human mitochondrial genome encoding for OXPHOS subunits. (**a**) Structure of mitochondrial genome. Text boxes represent human cancers that have a mutation found in mtdna genes. (**b**) Representation of mtdna vs. nuclear DNA (nDNA) encoded subunits of the OXPHOS chain. mtDNA encoded subunits are labeled (See Color Insert)

no introns and lacks histones. This, along with its close proximity to ROS produced through oxidative phosphorylation in the mitochondria, make mtDNA vulnerable to oxidative damage and mutations. In fact, the mutation frequency in mtDNA is approximately tenfold greater than that in nuclear DNA (Grossman and Shoubridge 1996; Johns 1995).

MtDNA within a single cell generally have identical sequences, which is described as homoplasmic. Heteroplasmy, or different mtDNA sequences within the same cell, can occur in response to somatic mutations (Chatterjee et al. 2006).

Since there can be thousands of copies of mtDNA within a single cell, mitochondrial pathologies usually arise only after a minimal threshold of heteroplasmy has been reached. That threshold is apparently different for different tissues and is dependent upon the energy status of a cell and its requirement for oxidative metabolism.

Mitochondria and Cancer

Metabolic Differences

The recent interest in the study of mitochondria has been fueled in large part by the recognition that genetic and/or biochemical alterations in this organelle are causative or contributing factors in a variety of human diseases, including cancer. A number of metabolic aberrations in cancer cells have been found to be specifically associated with mitochondrial metabolic function. Examples of these observations are listed in Table 2. These differences include altered enzyme activities or functions of proteins in the mitochondria, as well as altered membrane potential and composition, and expression of mitochondrial proteins. Electron transport chain complex activities involved in oxidative phosphorylation are known to be decreased in cancer vs. normal cells. Numerous differences in the molecular composition of the mitochondrial inner membrane between normal and cancer cells have also been reported. Some of these changes, such as increased cholesterol content, may be associated with increases in hexokinase, thus indicating a shift towards glycolytic metabolism (Campbell and Chan 2007). Polypeptide profiles of normal vs. cancer cells reveal a number of differences in the appearance and/or relative abundance of several proteins as well (Desouki et al. 2005). Stoichiometric mismatches in OXPOHOS complexes because of the altered expression of subunits have been hypothesized to lead to altered OXPHOS function. It is important to note that despite the large number of metabolic aberrations thus far identified, apparently none is common to all cancer cells.

Changes in Mitochondrial Genome

Mutations in mtDNA as well as alterations in mtDNA content have been reported in numerous cancers examined to date (Fig. 1c) (reviewed in Modica-Napolitano and Singh 2004; Modica-Napolitano et al. 2007). The D-loop region has been shown to be a mutational “hot spot” in human cancer. The incidence of somatic mutations in the D-loop region is found in all tumors examined to date and appears to be a universal feature of all cancers (Modica-Napolitano et al. 2007). These studies provide strong evidence that instability in the D-loop region of mtDNA may be

Table 2 Metabolic differences between normal and tumor cells

Decreased ATPase activity in hepatocellular carcinomas	Pedersen and Morris (1974) Capuano et al. (1997) Cuezva (1997)
Decreased cytochrome *c* oxidase activity in transformed, cultured cells, colonic adenocarcinoma and rat hepatoma cells	Modica-Napolitano and Touma (2000) Sun et al. (1981) Sun and Cederbaum (1980)
Decreased function of adenine nucleotide translocase in hepatoma liver mitochondria	Chan and Barbour (1983) Sul et al. (1979) Woldegiorgis and Shrago (1985)
Decreased protein level of β subunit of F_1 of ATPase leading to altered ATPase function in hepatocytes	Capuano et al. (1996) Capuano et al. (1997) Cuezva (1997)
Decreased expression of mtDNA encoded COX III subunit in colonic carcinoma biopsies and cultured cells	Heerdt et al. (1990)
Increased mitochondrial membrane potential in carcinoma cells	Summerhayes et al. (1982) Johnson et al. (1981) Modica-Napolitano and Aprille (1987)
Increased cholesterol content of the inner mitochondrial membrane	Campbell and Chan (2007)
Increased expression of mt and nuclear DNA encoded cytochrome *c* oxidase subunits in Zajdela hepatomas and prostate carcinoma	Luciakova and Kuzela (1992) Herrmann et al. (2003)
Increased expression of adenine nucleotide translocase 2 in renal tumor cells	Faure Vigny et al. (1996) Giraud et al. (1998)

involved in carcinogenesis of human cancers. Since the D-loop region contains regulating elements involved in mtDNA replication, mutation in the D-loop can affect the mtDNA copy number. Indeed, tumor-specific changes in the mtDNA copy number have been reported in human cancers. In one recent study investigating the mtDNA copy number in a variety of cancers, both increases and decreases in mtDNA content relative to non-malignant controls were observed for types of cancer (Lee et al. 2005). The mtDNA copy number has been shown to be increased in papillary thyroid carcinomas (Mambo et al. 2005) and during endometrial cancer development (Wang et al. 2005). Conversely, it has been reported that mtDNA content is reduced in breast and ovarian tumors relative to normal controls (Desouki et al. 2005). Additionally, mtDNA dimers (Clayton and Vinograd 1969) and large mtDNA mutations have been found in patients with leukemia (reviewed in the chapter "Mitochondria in Hematomology").

It has also been suggested that polymorphisms in mtDNA play a role in carcinogenesis and tumor progression. In a recent population-based study involving prostate cancer patients of European and African-American descent and benign controls without cancer, the frequency of COX I missense mutations was found to be significantly higher in prostate cancer patients than in the no-cancer controls. In some cases, the COX I sequence variants were thought to represent germline

mutations (Petros et al. 2005). Recent cybrid studies of polymorphisms in the ND6 subunit have shown an increased tumor metastatic potential (Ishikawa et al. 2008), and a polymorphism in the ND3 subunit shows increased anchorage-independent growth and resistance to apoptosis (Kulawiec and Singh 2008).

Changes in Nuclear Genome

Mutations in nuclear DNA that encode for mitochondrial proteins have also been shown to be connected to a tumorogenic phenotype (Fig. 3). Somatic and germline mutations in genes involved in the TCA cycle and oxidative phosphorylation have been identified in cancer patients (Tomlinson et al. 2002; Selak et al. 2005; Pollard et al. 2005; Douwes Dekkar et al. 2003; Neumann et al. 2004). Mutations in nuclear genes encoding mitochondrial proteins are not the only way that nuclear DNA may be involved with carcinogenesis. Mitochondrial dysfunction may also invoke mitochondria-to-nucleus retrograde responses in human cells (Singh 2004). Cybrid studies have revealed marked changes in the cellular proteome, including quantitative changes in the expression of several proteins in breast and ovarian tumors, which suggest that retrograde responsive genes may potentially function as tumor suppressor or oncogenes (Singh et al. 2005). These studies suggest that retrograde cross-talk between mitochondria and nucleus plays an important role in tumorigenesis.

A nuclear-encoded gene involved in the maintenance of the mitochondrial genome has also been implicated in cancer. Mutations in polymerase γ, a mtDNA polymerase, have been identified as associated with breast cancer (Singh et al. 2008). Both *in vitro* and *in vivo* studies have shown that mutations in the exonuclease domain of polymerase gamma lead to the accumulation of mitochondrial mutation, while mutations in the polymerase domain lead to the depletion of mtDNA (Spelbrink et al. 2000; Jazayeri et al. 2003; Lewis et al. 2007). Indeed, our studies provide direct evidence that the depletion of mtDNA leads to the transformation of breast epithelial cells (Kulawiec and Singh 2008).

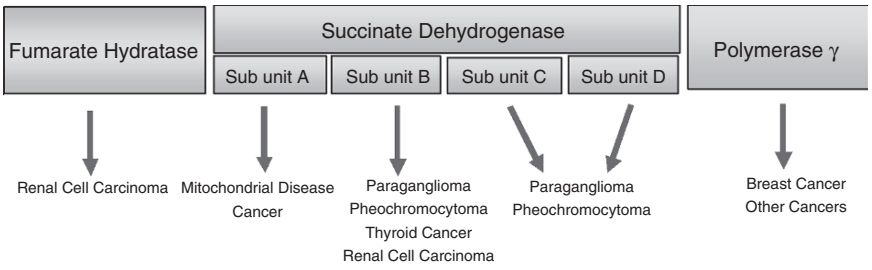

Fig. 3 Mutations found in nuclear encoded mitochondrial proteins that have been observed in human tumors

The Role of Mitochondrial ROS in Carcinogenesis

It has been suggested that ROS (i.e., superoxide, hydrogen peroxide, hydroxyl radical) may have a causative role in the progression of carcinogenesis (Ishii et al. 2005; Martinez-Cayuela 1995; Emerit 1994; Jackson and Loeb 2001; Christians et al. 1995). A large portion of ROS is produced in the mitochondria through metabolic processes (Martinez-Cayuela 1995; Cejas et al. 2004). Superoxide ($O_2^{\cdot -}$) is the primary radical produced. It is dismutated to hydrogen peroxide (H_2O_2), either spontaneously or enzymatically, through superoxide dismutase (SOD). Hydrogen peroxide can undergo Fenton chemistry to form the highly reactive hydroxyl radical (Martinez-Cayuela 1995). At normal cellular levels, ROS plays a role in cell-signaling normal functions in the cell. However, when ROS is in excess, damage to cellular components, including protein, lipids, and DNA, occurs. This damage may be done by ROS itself or propagated through long-lived clastogenic factors (Emerit 1994). Lipid peroxidation leads to the formation of aldehydes such as 4-hydroxynonenol (4-HNE) and malodialdehyde (MDA), which have been shown to induce DNA damage at high levels (Cejas et al. 2004; Liu et al. 2003; Marnett 1999; Nair et al. 1999; Yen et al. 1994). These factors explain how short-lived free radical species in the mitochondria are capable of damaging cellular components in the cell.

The electron transport chain is the major source of superoxide production within the cell. In normal respiring cells, up to 2% of the O_2 in oxidative phosphorylation undergoes a one-electron reduction to produce superoxide (Boveris and Chance 1973); however, this may be much higher in cancer cells (Turrens 1997, 2003; Kowaltowski and Vercesi 1998). It has been well known that superoxide production comes from complex I, leaking electrons into the matrix of the mitochondria, and complex III, leaking electrons into the matrix as well as the inner membrane space. Although it is believed that complexes I and III of the electron transport chain (ETC) are major sources of the electron leak in normal cells (Boveris and Chance 1976; Turrens and Boveris 1980), recent studies have suggested that a defective complex II is also able to cause the univalent reduction of oxygen to superoxide (Ishii et al. 2005; Slane et al. 2006). Also, it has been proposed that electrons from succinate in complex II may go through reverse electron transport to NAD^+ in complex I and leak there (reviewed in Brand et al. 2004).

Implications of O_2^- and H_2O_2 production by the mitochondrial ETC range from cell signaling mechanisms to oxidative damage. Increased ROS production from the ETC may lead to an environment of oxidative stress within the mitochondria, which, in turn, may result in damage to DNA or cellular components. It has been shown that ROS from the ETC causes genomic instability and increased mutation rates (Spitz et al. 2004; Slane et al. 2006; Yakes and Van Houten 1997; Suzuki et al. 2002). These damages may lead to carcinogenesis, accelerated aging, and other degenerative diseases. For example, an increase in oxidative phosphorylation is the initial event that occurs early in the malignant transformation of peripheral zone prostate cells. The increased respiratory activity is thought to then induce a cascade of events, including increased ROS production, increased mtDNA mutations,

compromised respiratory activity, and further ROS production, thus setting in motion a cycle of oxidative stress that may contribute to the etiology and pathogenesis of prostate cancer (Dakubo et al. 2006).

It has been hypothesized that oxidative stress resulting from chronic inflammation may be linked to carcinogenesis. For example, one recent study observed that mtDNA mutations in subjects with chronic inflammation because of ulcerative colitis had, in 8-hydroxy-2-deoxyguanosine, a DNA lesion caused by oxidative damage (Nishikawa et al. 2005). Additionally, there is evidence that oxidative stress associated with chronic inflammation may also be involved in the pathogenesis of prostate cancer (Platz and De Marzo 2004; De Marzo et al. 1999). In support of this hypothesis, it has recently been demonstrated that defects in oxidative phosphorylation lead to an increase in superoxide-producing NADPH oxidase (NOX1) in breast and ovarian cancer (Desouki et al. 2005).

Cells are equipped with antioxidant enzymes that detoxify ROS and protect the cell from oxidative damage. SOD catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen (McCord and Fridovich 1969; Oberley 2001). Hydrogen peroxide is detoxified into water and oxygen by catalase (Kono and Fridovich 1982; Aebi 1984), while hydrogen peroxide and other hydroperoxides can be detoxified by glutathione peroxidase (GPx) in the mitochondria and in the cytosol (Chu et al. 1993, 2004; Sies 1999). All three of these antioxidant enzymes have been shown to have altered levels in tumor cells as compared with their normal tissue counterpart. In the majority of these cases, the cancer cells were found to be deficient in antioxidant enzymes (Oberley and Buettner 1979; Oberley 2001; Chu et al. 2004).

The mitochondrial form of SOD, manganese SOD (MnSOD), has been suggested to be a tumor-suppressor gene in that it inhibits the progression of cancer by detoxifying superoxides (Oberley 2001). Mutations in the promoter region and epigenetic regulation have led to the decreased expression of MnSOD in cancer cells. In fact, a polymorphism in the mitochondrial import signal has been associated with an increased risk of prostate cancer. In addition, inadequate selenium levels, necessary for the activity of some GPx, have been found in cancer patients (Klein et al. 2003).

Mitochondrial Stress Signaling

Alterations in gene expression and metabolic function in cancer cells may not only have an effect on energy and ROS production. Signaling pathways that lead to apoptosis and gene regulation have also been shown to be affected. The gene expression between normal and cancer cells includes the anti-apoptotic oncogenes encoding Bcl-2 and Bcl-XL, and genes encoding the peripheral benzodiazepin receptor (PBR), the PBR-associated protein Prax-1 and mitochondrial creatine kinase (Reed 1994, 1997; O'Gorman et al. 1997; Kroemer 1997; Schiemann et al. 1998; Venturini et al. 1998; Galiegue et al. 1999; Beurdeley-Thomas et al. 2000).

The expression of BAX, a proapoptotic, inner mitochondrial membrane protein, is also reduced in some cancer cell-lines (Rampino et al. 1997; Brimmell et al. 1998).

Another pathway involved in cancer progression, which is affected by mitochondrial metabolism, is the stabilization of HIF-1α. HIF-1α is a transcription factor that is degraded in the presence of O_2. Prolyl hydroxylase (PHD) puts hydroxyl groups on the oxygen-dependent domain of HIF-1α. These hydroxyl groups are recognized by a ubiquitin ligase, Von Hippel Lindau protein, and HIF-1α is tagged for degradation. Upon stabilization under hypoxic conditions, HIF-1α enters the nucleus and binds to HIF-1β to induce expression on pro-angiogenic and glycolytic genes (Pugh and Ratcliffe 2003). An accumulation of succinate because of decreased activities of SDH and/or FH has been shown to lead to the stabilization of HIF-1α and an increased expression of pro-angiogenesis gene, VEGF (Selak et al. 2005). In the process of hydroxylating HIF-1α protein, PHD converts α-ketoglutarate into succinate. An excess of intracellular succinate may work through product inhibition to stabilize HIF-1α under normoxic conditions (Selak et al. 2005).

Pollard and colleagues showed that paraganglioma tumors with mutations in SDHB, C, and D also had an increase in HIF-1α protein, as determined by immunohistochemistry on paraffin-embedded tissue (Pollard et al. 2005). Gimenez-Roqueplo and colleagues also observed elevated VEGF expression in pheochromocytoma tissue that harbored a nonsense mutation in SDHD (2001). Increased activation of HIF-1α in tumors with mutations in SDH and FH nuclear genes would be expected to give the tumor a growth advantage through increased vascularization, allowing for adequate delivery of oxygen and glucose. Also, HIF-1α accumulation promotes a metabolic shift glycolysis (Covello and Simon 2004; Gao et al. 2002).

Most recently, the link between altered energy metabolism and cancer has been strengthened by the discovery that the tumor-suppressor p53 regulates oxidative phosphorylation activity. These results suggest that a loss of p53 results in a metabolic shift away from aerobic respiration toward production of glycolytic ATP, i.e., the Warburg effect (Matoba et al. 2006).

Posttranslational modifications of electron transport chain proteins, such as glutathionylation and nitrosylation, have been hypothesized to play an important role in mitochondrial regulation and signaling. Glutathionylation occurs when glutathione disulfide (GSSG) undergoes a disulfide exchange reaction with protein sulfhydryls (PSH) to form mixed protein disulfides of glutathione (PSSG), a mixed protein disulfide. Another type of posttranslational modification found in ETC proteins is S-nitrosylation in which an NO group is added to a cysteine residue. Formation of *S*-nitrosothiols (SNO) has been demonstrated with ONOO− and NO donors, such as *S*-nitrosoglutathione (GSNO) and Deta-NONOate. The process of transferring NO^+ from a nitrosothiol, such as GSNO, is known as transnitrosation (Borutaite et al. 2000).

Complex I has been shown to be susceptible to both glutathionylation at the 51 and 75 kDa subunits (Taylor et al. 2003; Chen et al. 2007a, b) and S-nitrosylation at the 75 kDa subunit (Borutaite et al. 2000; Clementi et al. 1998; Burwell et al. 2006).

Complex II has been shown to be glutathionalyated at subunit A (Chen et al. 2007a, b). These modifications led to decreased complex I activity and altered ROS levels. The S2 subunit of complex IV has been shown to be nitrosylated. These residues are involved in the redox centers of complex IV, whereupon nitrosylation of the copper may be released, leading to decreased activity (Zhang et al. 2005).

Both glutathionylation and S-nitrosylation of ETC protein may result in mitochondrial signaling. It has been speculated that the glutathionylation of complex I may be a signal to indicate the GSH/GSSG redox state of the mitochondria (Chen et al. 2007a, b). Glutathionylation of complex I causes $O_2^{\cdot -}$ production from the enzyme to increase. This $O_2^{\cdot -}$ is dismutated into H_2O_2, either spontaneously or through MnSOD, and may act as a secondary messenger in the cytosol to activate NF-κB, JNK, p53, and Ras (Taylor et al. 2003). On the contrary, the reversal of glutathionylation of complex II was observed after I/R decreased the $O_2^{\cdot -}$ production from complex II in an effort to dampen oxidative injury following I/R. The S-nitrosylation of complex I was hypothesized to be a signal to decrease ROS production in the mitochondria under conditions of stress by stopping electrons from entering the ETC and leaking from complex III. Burwell et al. provided supporting evidence for this hypothesis by showing that ischemic preconditioning of rat hearts increased the amount SNO detected in complex I (Burwell et al. 2006). Mitochondrial respiration has also been shown to be governed by NO in the mitochondria by the inhibition of complex IV (Cleeter et al. 1994; Poderoso et al. 1996; Brookes and Darley-Usmar 2002).

Mitochondria as Biomarkers for Cancer

A number of recent studies suggest that the functional significance of mtDNA mutations and depletions may be a useful marker of tumorigenesis and/or tumor progression. For example, the presence of somatic D-loop mutations appears to be a factor of poor prognosis in colorectal cancer patients, and may be a factor of resistance to fluorouracil-based adjuvant chemotherapy in stage II cancers (Lievre et al. 2005). Additionally, there is epidemiological evidence that the mtDNA 10398A polymorphism, which alters the structure of electron transport complex I, is associated with increased risk for breast cancer in African-American women (Canter et al. 2005). It has been hypothesized that the germline COX I mutation may increase the incidence of prostate cancer in African-American men (Petros et al. 2005). The abundance and homoplasmic nature of mitochondria make mtDNA an attractive molecular marker of cancer (Polyak et al. 1998; Singh 1998; Singh et al. 1999). Mutant mtDNA is readily detectable in urine, blood, and saliva samples from patients with bladder, head and neck, and lung cancers (Fliss et al. 2000; Jiang et al. 2005). Recently, rapid- and high-throughput sequencing protocols have been developed to detect mtDNA sequence variants in patient tumor and blood samples (Jakupciak et al. 2005).

Mitochondria as Targets for Chemotherapy

The differences described above in mitochondrial structure and function between normal cells and cancer cells not only offer clinical markers for diagnoses, but may also provide targets for chemotherapy (Weissig and Torchilin 2001; Modica-Napolitano and Singh 2002). Utilizing differences such as increased mitochondrial membrane potential may help selectively target molecules such as delocalized lipohilic cations (DLCs), which exhibit a cytotoxic effect in the mitochondria (Modica-Napolitano et al. 1996; Weisberg et al. 1996). Compounds such as these that are selectively taken up by the mitochondria of transformed cells may be used in combination with photochemotherapy (PCT). PCT is a cancer treatment that involves light activation of a photoreactive drug, or photosensitizer (Dougherty et al. 1985; Wilson and Jeeves 1987; Powers 1988; Modica-Napolitano et al. 1990). There has been considerable interest in PCT as a form of treatment for neoplasms of the skin, lung, breast, bladder, brain or any other issue accessible to light transmitted either through the body surface or internally via fiber-optic endoscopes. In response to localized photoirradiation, the photosensitizer can be converted to a more reactive and highly toxic species, thus enhancing the selective toxicity to carcinoma cells and providing a means of highly specific tumor-cell killing without injury to normal cells.

Other chemotherapeutic strategies target protein-import machinery to deliver macromolecules to mitochondria and specific mitochondrial membrane proteins to induce membrane permeabilization and, ultimately, apoptosis (Costantini et al. 2000). In addition, drug therapies that used the cancer cell's increased glycolysis are being tested with conventional therapies. Compounds such as 2-deoxyglucose, a competitive inhibitor of glycolysis, and low carbohydrate/high-protein diets are currently being explored in combination with conventional therapies (Lin et al. 2003; Simons et al. 2007; Coleman et al. 2008; Freedland et al. 2008; Otto et al. 2008). Also, the small molecule dichloroacetate (DCA), a pyruvate dehydrogenase kinase inhibitor, shifts the metabolism of the cell from glycolosis to glucose oxidation, leading to a decrease in tumor growth and increased cancer cell apoptosis (Bonnet et al. 2007). This small molecule could potentially be used to selectively kill cancer cells that are dependent on glycolysis while having little side-effects on normal cells.

Attempts are also being made to develop mitochondriotropic drug and DNA delivery systems. Recent data demonstrates that conventional liposomes can be rendered mitochondria specific via the attachment of known mitochondriotropic residues to the liposomal surface (Boddapati et al. 2005). Furthermore, DQAsomes prepared from derivatives of the self-assembling mitochondriotropic bola-amphiphile dequalinium chloride have been shown to bind and transport oligonucleotides as well as plasmid DNA conjugated to a mitochondrial leader sequence (MLS) to mitochondria in living mammalian cells and to release DNA on contact with mitochondrial membranes (D'Souza et al. 2005). The long-term therapeutic goal

of this type of research is to someday create mitochondria-specific vehicles that will effectively deliver drugs or mtDNA into the organelle to destroy dysfunctional mitochondria or replenish mitochondria with healthy copies of the genome.

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References

Aebi, H. 1984. Catalase in vitro. Methods Enzymol. 105:121–126

Aufderheide, K.J. 1980. Mitochondrial associations with specific microtubular components of the cortex of Tetrahymena thermophila. II. Response of the mitochondrial pattern to changes in the microtubule pattern. J. Cell. Sci. 142:247–260

Beurdeley-Thomas, A., Miccoli, L., Oudard, S., Dutrillaux, B., and Poupon, M.F. 2000. The peripheral benzodiazepine receptors: A review. J. Neurooncol. 46:45–56

Bianchi, N.O., Bianchi, M.S., Richard, S.M. 2001. Mitochondrial genome instability in human cancers. Mutat. Res. 488:9–23

Boddapati, S.V., Tongcharoensirikul, P., Hanson, R.N., D'Souza, G.G., Torchilin, V.P., Weissig, V. 2005. Mitochondriotropic liposomes. J. Liposome Res. 15:49–58

Bonnet, S., Archer, S.L., Allalunis-Turner, J., Haromy, A., Beaulieu, C., Thompson, R., Lee, C.T., Lopaschuk, G.D., Puttagunta, L., Bonnet, S., Harry, G., Hashimoto, K., Porter, C.J., Andrade, M.A., Thebaud, B., Michelakis, E.D. 2007. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. Cancer Cell. 11:37–51

Borutaite, V., Budriunaite, A., and Brown, G.C. 2000. Reversal of nitric oxide-, peroxynitrite- and *S*-nitrosothiol-induced inhibition of mitochondrial respiration or complex I activity by light and thiols. Biochim. Biophys. Acta 1459:405–412

Boveris, A., and Chance, B. 1973. The mitochondrial generation of hydrogen peroxide. Biochem. J. 134:707–716

Brand, M.D., Affouritt, C., Esteves, T.C., Green, K., Lambert, A.J., Miwa, S., Pakay, J.L., and Parker, N. 2004. Free Radic. Biol. Med. 37:755–767

Brimmell, M., Mendiola, R., Mangion, J., and Packham, G. 1998. BAX frameshift mutations in cell lines derived from human haemopoietic malignancies are associated with resistance to apoptosis and microsatellite instability. Oncogene 16:1803–1812

Brookes, P.S., and Darley-Usmar, V.M. 2002. Hypothesis: The mitochondrial NO signalling pathway, and the transduction from nitrosative to oxidative cell signals: An alternative function for cytochrome *c* oxidase. Free Radic. Biol. Med. 32:370–374

Burwell, L.S., Nadtochiy, S.M., Tompkins, A.J., Young, S., and Brookes, P.S. 2006. Direct evidence for S-nitrosation of mitochondrial complex I. Biochem. J. 394:627–634

Campbell, A.M., and Chan, S.H.P. 2007. The voltage dependent anion channel affects mitochondrial cholesterol distribution and function. Arch. Biochem. Biophys. 466:203–210

Canter, J.A., Kallianpur, A.R., Parl, F.F., Millikan, R.C. 2005. Mitochondrial DNA G10398A polymorphism and invasive breast cancer in African-American women. Cancer Res. 65:8028–8033

Capuano, F., Guerrieri, F., and Papa, S. 1997. Oxidative phosphorylation enzymes in normal and neoplastic cell growth. J. Bioenerg. Biomembr. 29:379–384

Capuano, F., Varone, D., D'Eri, N., Russo, E., Tommasi, S., Montemurro, S., Prete, F., and Papa, S. 1996. Oxidative phosphorylation and F(0)F(1) ATP synthase activity of human hepatocellular carcinoma. Biochem. Mol. Biol. Int. 38:1013–1022

Carafoli, E. 1980. Mitochondria and disease. Molec. Aspects Med. 3:295–429

Cejas, P., Casado, E., Belda-Iniesta, C., De Castro, J., Espinosa, E., Redondo, A., Sereno, M., Garcia-Cabezas, M.A., Vara, J.A.F., Domingues-Caceres, A., Rosario, P., and Gonzalez-Baron, M. 2004. Implications of oxidative stress and cell membrane lipid peroxidation in human cancer. Cancer Causes Control 15:707–719

Chan, S.H., and Barbour, R.L. 1983. Adenine nucleotide transport in hepatoma mitochondria. Characterization of factors influencing the kinetics of ADP and ATP uptake. Biochim. Biophys. Acta 723:104–113

Chatterjee, A., Mambo, E., Sidransky, D. 2006. Mitochondrial DNA mutations in human cancer. Oncogene 25:4663–4674

Chen, Y.R., Chen, C.L., Pfeiffer, D.R., and Zweier, J.L. 2007a. Mitochondrial complex II in the post-ischemic heart: Oxidative injury and the role of protein S-glutathionylation. J. Biol.Chem. 282:32640–32654

Chen, C.L., Zhang, L., Yeh, A., Chen, C.A., Green-Church, K.B., Zweier, J.L., and Chen, Y.R. 2007b. Site-specific S-glutathiolation of mitochondrial NADH ubiquinone reductase. Biochemistry 46:5754–5765

Christians, F.C., Newcomb, T.G., and Loeb, L.A. 1995. Potential sources of multiple mutations in human cancers. Prev. Med. 24:329–332

Chu, F.F., Doroshow, J.H., and Esworthy, R.S. 1993. J. Biol. Chem. 4:2571–2576

Chu, F.F., Esworthy, R.S., and Doroshow, J.H. 2004. Free Radic. Biol. Med. 36:1481–1495

Clayton, D.A., and Vinograd, J. 1969. Complex mitochondrial DNA in leukemic and normal human myeloid cells. Proc. Natl. Acad. Sci. USA. 62:1077–1084

Cleeter, M.W.J., Cooper, J.M., Darley-Usmar, V.M., Moncada, S., and Schapira, A.H.V. 1994. Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. FEBS Lett. 345:50–54

Clementi, E., Brown, G.C., Feelish, M., and Moncada, S. 1998. Persistent inhibition of cell respiration by nitric oxide: Crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. Proc. Nat. Acad. Sci. USA 95:7631–7636

Covello, K.L., and Simon, M.C. 2004. HIFs, hypoxia, and vascular development. Curr. Topics Dev. Biol. 62:37–54

Coleman, M.C., Asbury, C.R., Daniels, D., Du, J., Aykin-Burns, N., Smith, B.J., Li, L., Spitz, D. R., Cullen, J.J. 2008. 2-Deoxy-D-glucose causes cytotoxicity, oxidative stress, and radiosensitization in pancreatic cancer. Free Radic. Biol. Med. 44:322–331

Costantini, P., Jacotot, E., Decaudin, D., Kroemer, G. 2000. Mitochondrion as a novel target of anticancer chemotherapy. J. Natl. Cancer Inst. 92:1042–1053

Cuezva , J.M., Ostronoff, L.K., Ricart, J., López de Heredia, M., Di Liegro, C.M., and Izquierdo, J.M. 1997. Mitochondrial biogenesis in the liver during development and oncogenesis. J. Bioenerg. Biomembr. 29:365–377

Dakubo, G.D., Parr, R.L., Costello, L.C., Franklin, R.B., Thayer, R.E. 2006. Altered metabolism and mitochondrial genome in prostate cancer. J. Clin. Pathol. 59:10–16

De Marzo, A.M., Marchi, V.L., Epstein, J.I., Nelson, W.G. 1999. Proliferative inflammatory atrophy of the prostate: Implications for prostatic carcinogenesis. Am. J. Pathol. 155:1985–1992

Desouki, M.M., Kulawiec, M., Bansal, S., Das, G.M., Singh, K.K. 2005. Cross talk between mitochondria and superoxide generating NADPH oxidase in breast and ovarian tumors. Cancer Biol. Ther. 4:1367–1373

Dougherty, T.J., Weishaupt, K.R., and Boyle, D.G. 1985. Photodynamic Sensitizers. J.B. Lipincott, Philadelphia, PA, USA

Douwes Dekker, P.B., Hogendoorn, P.C.W., Kuipers-Dijkshoorn, N., Prins, F.A., van Duinen, S. G., Taschner, P.E.M., van der Mey, A.G.L., and Cornelisse, C.J. 2003. SDHD mutations in

head and neck paragangliomas result in destabilization of complex II in the mitochondrial respiratory chain with loss of enzymatic activity and abnormal mitochondrial morphology. J. Path. 201:480–486

D'Souza, G.G., Boddapati, S.V., Weissig, V. 2005. Mitochondrial leader sequence-Plasmid DNA conjugates delivered into mammalian cells by DQAsomes co-localize with mitochondria. Mitochondrion 5:352–358

Emerit, I. 1994. Reactive oxygen species, chromosome mutation, and cancer: Possible role of clastogensic factors in carcinogenesis. Free Radic. Biol. Med. 16:99–109

Faure Vigny, H., Heddi, A., Giraud, S., Chautard, D., and Stepien, G. 1996. Expression of oxidative phosphorylation genes in renal tumors and tumoral cell lines. Mol. Carcinog. 16:165–172

Fliss, M.S., Usadel, H., Caballero, O.L., Wu, L., Buta, M.R., Eleff, S.M., Jen, J., Sidransky, D. 2000. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science 287:2017–2019

Freedland, S.J., Mavropoulos, J., Wang, A., Darshan, M., Demark-Wahnefried, W., Aronson, W. J., Cohen, P., Hwang, D., Peterson, B., Fields, T., Pizzo, S.V., Isaacs, W.B. 2008. Carbohydrate restriction, prostate cancer growth, and the insulin-like growth factor axis. Prostate 68:11–19

Galiegue, S., Jbilo, O., Combes, T., Bribes, E., Carayon, P., Le Fur, G., and Casellas, P. 1999. Cloning and characterization of PRAX-1. A new protein that specifically interacts with the peripheral benzodiazepine receptor. J. Biol. Chem. 274:2938–2952

Gao, N., Ding, M., Zheng, J.Z., Zhang, Z., Leonard, S.S., Liu, K.J., Shi, X., Jiang, B.H. 2002. Vanadate-induced expression of hypoxia-inducible factor 1 alpha and vascular endothelial growth factor through phosphatidylinositol 3-kinase/Akt pathway and reactive oxygen species. J. Biol. Chem. 277:31963–31971

Gibson, B.W. 2005. The human mitochondrial proteome: Oxidative stress, protein modifications and oxidative phosphorylation. Int. J. Biochem. Cell Biol. 37:927–934

Gimenez-Roqueplo, A.P., Favier, J., Rustin, P., Mourad, J.J., Plouin, P.F., Corvol, P., Rotig, A., and Jeunemaitre, X. 2001. The R22X mutation of the SDHD gene in hereditary paraganglioma abolishes the enzymatic activiy of Complex II in the mitochondrial respiratory chain and activities the hypoxia pathway. Am. J. Hum. Genet. 69:1186–1197

Giraud, S., Bonod-Bidaud, C., Wesolowski-Louvel, M., and Stepien, G. 1998. Expression of human ANT2 gene in highly proliferative cells: GRBOX, a new transcriptional element, is involved in the regulation of glycolytic ATP import into mitochondria. J. Mol. Biol. 281:409–418

Grossman, L.I., and Shoubridge, E.A. 1996. Mitochondrial genetics and human disease. Bioessays 18:983–991

Heerdt, B.G., Halsey, H.K., Lipkin, M., and Augenlicht, L.H. 1990. Expression of mitochondrial cytochrome *c* oxidase in human colonic cell differentiation, transformation, and risk for colonic cancer. Cancer Res. 50:1596–1600

Herrmann, P.C., Gillespie, J.W., Charboneau, L., Bischel, V.E., Paweletz, C.P., Calvert, V.S., Kohn, E.C., Emmert-Buck, M.R., Liotta, L.A., and Petricoin, E.F. 2003. Mitochondrial proteome: Altered cytochrome *c* oxidase subunit levels in prostate cancer. Proteomics 3:1801–1810

Ishii, T., Yasuda, K., Akatsuka, A., Hino, O., Hartman, P.S., and Ishii, N. 2005. A mutation in the SDHC gene of Complex II increases oxidative stress, resulting in apoptosis and tumorigenesis. Cancer Res. 65:203–209

Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguchi, A., Imanishi, H., Nakada, K., Honma, Y., Hayashi, J. 2008. ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. Science. 320:661–664

Jackson, A.L., and Loeb, L.A. 2001. The contribution of endogenous sources of DNA damage to the mutliple mutations in cancer. Mutat. Res. 477:7–21

Jakupciak, J.P., Wang, W., Markowitz, M.E., Ally, D., Coble, M., Srivastava, S., Maitra, A., Barker, P.E., Sidransky, D., O'Connell, C.D. 2005. Mitochondrial DNA as a cancer biomarker. J Mol. Diagn. 7:258–267

Jazayeri, M., Andreyev, A., Will, Y., Ward, M., Anderson, C.M., Clevenger, W. 2003. Inducible expression of a dominant negative DNA polymerase-gamma depletes mitochondrial DNA and produces a rho0 phenotype. J. Biol. Chem. 278:9823–9830

Jiang, W.W., Masayesva, B., Zahurak, M., Carvalho, A.L., Rosenbaum, E., Mambo, E., Zhou, S., Minhas, K., Benoit, N., Westra, W.H., Alberg, A., Sidransky, D., Koch, W., Califano, J. 2005. Increased mitochondrial DNA content in saliva associated with head and neck cancer. Clin Cancer Res 11:2486–2491

Johns, D.R. 1995. Seminars in medicine of the Beth Israel Hospital, Boston. Mitochondrial DNA and disease. New Engl. J. Med. 333:638–644

Johnson, L.V., Walsh, M.L., Bockus, B.J., Chen, L.B. 1981. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. J. Cell Biol. 88:526–535

Klein, E.A., Lipman, S.M., Thompson, I.M., Goddman, P.J., Albanes, D., Taylor, P.R., and Coltman, C. 2003. World J. Urol. 21:21–27

Kono, Y., and Fridovich, I. 1982. Superoxide radical inhibits catalase. J. Biol. Chem. 257:5751–5754

Kowaltowski, A.L., and Vercesi, A.E. 1998. Mitochondrial damage induced by conditions of oxidative stress. Free Radic. Biol. Med. 26:463–471

Kroemer, G. 1997. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat. Med. 3:614–620

Kroemer, G., Galluzzi, L., and Brenner, C. 2007. Mitochondrial membrane permealization in cell death. Physiol. Rev. 87:99–163

Kulawiec, M., Safina, A., Dosouki, M., Matsi, I., Bakin, A., and Singh, K.K. 2008. Tumorigenic transformation of human breast epithelial cells induced by mitochondrial DNA depletion. Cancer Biol. Therapy. 263:4450–4459

Lee, H.C., Yin, P.H., Lin, J.C., Wu, C.C., Chen, C.Y., Wu, C.W., Chi, C.W., Tam, T.N., Wei, Y.H. 2005. Mitochondrial genome instability and mtDNA depletion in human cancers. Ann. NY Acad. Sci. 1042:109–122

Lewis, W., Day, B.J., Kohler, J.J., Hosseini, S.H., Chan, S.S., Green, E.C., Haase, C.P., Keebaugh, E.S., Long, R., Ludaway, T., Russ, R., Steltzer, J., Tioleco, N., Santoianni, R., Copeland, W.C. 2007. Decreased mtDNA, oxidative stress, cardiomyopathy, and death from transgenic cardiac targeted human mutant polymerase gamma. Lab Invest. 87:326–335

Lin, X., Zhang, F., Bradbury, C.M., Kaushal, A., Li, L., Spitz, D.R., Aft, R.L., Gius, D. 2003. 2-Deoxy-D-glucose-induced cytotoxicity and radiosensitization in tumor cells is mediated via disruptions in thiol metabolism. Cancer Res. 63:3413–3417

Lievre, A., Chapusot, C., Bouvier, A.M., Zinzindohoue, F., Piard, F., Roignot, P., Arnould, L., Beaune, P., Faivre, J., Laurent-Puig, P. 2005. Clinical value of mitochondrial mutations in colorectal cancer. J. Clin. Oncol. 23:3517–3525

Liu, C.S., Tsai, C.S., Kuo, C.L., Chen, H.W., Lii, C.K., Ma, Y.S., and Wei, Y.H. 2003. Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes. Free Radic. Res. 37:307–1317

Luciakova, K., and Kuzela, S. 1992. Increased steady-state levels of several mitochondrial and nuclear gene transcripts in rat hepatoma with a low content of mitochondria. Eur. J. Biochem. 205:1187–1193

Mambo, E., Chatterjee, A., Xing, M., Tallini, G., Haugen, B.R., Yeung, S.C., Sukumar, S., Sidransky, D. 2005. Tumor-specific changes in mtDNA content in human cancer. Int. J. Cancer 116:920–924

Marnett, L.J. 1999. Lipid peroxidation-DNA damage by malondialdehyde. Mutat. Res. 424:83

Martinez-Cayuela, M. 1995. Oxygen free radicals and human disease. Biochimie 77:47–161

Matoba, S., Kang, J.G., Patino, W.D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P.J., Bunz, F., Hwang, P.M. 2006. p53 regulates mitochondrial respiration. Science 312:1650–1653

McCord, J.M., and Fridovich, I. 1969. An enzymtic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244:6049–6055

Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature 191:144–148

Modica-Napolitano, J.S., and Aprille, J.R. 1987. Basis for the selective cytotoxicity of rhodamine 123. Cancer Res. 47:4361–4365

Modica-Napolitano, J.S., Joyal, J.L., Ara, G., Oseroff, A.R., Aprille, J.R. 1990. Mitochondrial toxicity of cationic photosensitizers for photochemotherapy. Cancer Res. 50:7876–7881

Modica-Napolitano, J.S., Koya, K., Weisberg, E., Brunelli, B.T., Li, Y., Chen, L.B. 1996. Selective damage to carcinoma mitochondria by the rhodacyanine MKT-077. Cancer Res. 56:544–550

Modica-Napolitano, J.S., Kulawiec, M., and Singh, K.K. 2007. Mitochondria and human cancer. Curr. Molec. Med. 7:1–11

Modica-Napolitano, J.S., and Touma, S.E. 2000. Functional differences in mitochondrial enzymes from normal epithelial and carcinoma cells. In Mitochondrial Dysfunction in Pathogenesis, A Keystone Symposium, 15–20 January, Santa Fe, NM, USA, p. 64. Keystone Symposia, Silverthorne, CO, USA

Modica-Napolitano, J.S., and Singh, K.K. 2002. Mitochondria as targets for detection and treatment of cancer. Exp. Rev. Mol. Med. 11 April, http://www.expertreviews.org/02004453h.htm

Modica-Napolitano, J., and Singh, K.K. 2004. Mitochondrial dysfunction in cancer. Mitochondrion 4:755–762

Nair, J., Barbin, A., Velic, I., and Bartsch, H. 1999. Etheno DNA-base adducts from endogenous reactive species. Mutat. Res. 424:59

Nelson, D.L., and Cox, M.M. 2000. Lehninger Principles of Biochemistry. Worth Publishers

Neumann, H.P., Pawlu, C., Peczkowaska, M., Bausch, B., McWinney, S.R., Muresan, M., Cuchta, M., Franke, G., Klisch, J., Bley, T.A., et al. 2004. Distinct clinical features of paraglioma syndromes associated with SDHB and SDHD gene mutations. J. Am. Med. Assoc. 292:943–951

Nishikawa, M., Oshitani, N., Matsumoto, T., Nishigami, T., Arakawa, T., Inoue, M. 2005. Accumulation of mitochondrial DNA mutation with colorectal carcinogenesis in ulcerative colitis. Br. J. Cancer 93:331–337

Oberley, L.W. 2001. Antioxidant enzyme levels as a function of growth state in cell culture. Antioxidants Redox Signall. 3:461–472

Oberley, L.W., and Buettner, G.R. 1979. Role of superoxide dismutase in cancer: A review. Cancer Res. 39:1141–1149

O'Gorman, E., Beutner, G., Dolder, M., Koretsky, A.P., Brdiczka, D., and Wallimann, T. 1997. The role of creatine kinase in inhibition of mitochondrial permeability transition. FEBS Lett. 414:253–257

Otto, C., Kaemmerer, U., Illert, B., Muehling, B., Pfetzer, N., Wittig, R., Voelker, H.U., Thiede, A., Coy, J.F. 2008. Growth of human gastric cancer cells in nude mice is delayed by a ketogenic diet supplemented with omega-3 fatty acids and medium-chain triglycerides. BMC Cancer 8:122

Pedersen, P.L., and Morris, H.P. 1974. Uncoupler-stimulated adenosine triphosphatase activity. Deficiency in intact mitochondria from Morris hepatomas and ascites tumor cells. J. Biol. Chem. 249:3327–3334

Pedersen, P.L. 1978. Tumor mitochondria and the bioenergetics of cancer cells. Prog. Exp. Tumor Res. 22:190–274

Petit, P.X., and Kroemer, G. 1998. Mitochondrial regulation of apoptosis. In Mitochondrial DNA Mutations in Aging, Disease and Cancer (Singh, K.K., ed.), pp. 147–165. Springer-Verlag, Berlin, Germany

Petros, J.A., Baumann, A.K., Ruiz-Pesini, E., Amin, M.B., Sun, C.Q., Hall, J., Lim, S., Issa, M.M., Flanders, W.D., Hosseini, S.H., Marshall, F.F., Wallace, D.C. 2005. mtDNA mutations increase tumorigenicity in prostate cancer. PNAS 102:719–724

Platz, E.A., and De Marzo, A.M. 2004. Epidemiology of inflammation and prostate cancer. J. Urol. 171:S36–40

Poderoso, J.J., Carreras, M.C., Lisdero, C., Riobo, N., Schopfer, F., and Boveris, A. 1996. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. Arch. Biochem. Biophys. 328:85–92

Pollard, P.J., Briere, J.J., Alam, N.A., Barwell, J., Barclay, E., Wortham, N.C., Hunt, T., Mitchell, M., Olpin, S., Moat, S.J., Hargreaves, I.P., Heales, S.J., Chung, Y.L., Griffiths, J.R., Dalgleish, A., McGrath, J.A., Gleeson, M.J., Hodgson, S.V., Poulsom, R., Rustin, P., and Tomlinson, I.P. M. 2005. Accumulation of Krebs cycle intermediates and over-expression of HIF1 in tumours which result from germline *FH* and *SDH* mutations. Hum. Mol. Genet. 14:2231–2239

Polyak, K., Li, Y., Zhu, H., Lengauer, C., Willson, J.K., Markowitz, S.D., Trush, M.A., Kinzler, K. W., Vogelstein, B. 1998. Somatic mutations of the mitochondrial genome in human colorectal tumours Nat. Genet. 20:291–293

Powers, S.K. 1988. In Application of Lasers in Neurosurgery (Cerullo, L.J. ed.), pp. 137–155. Year Book Medical Publishers, Chicago, IL, USA

Pugh, C.W., and Ratcliffe, P.J. 2003. Regulation of angiogenesis by hypoxia: Role of the HIF system. Nat. Med. 9:677–684

Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J.C., and Perucho, M. 1997. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. Science 275:967–969

Reed, J.C. 1994. Bcl-2 and the regulation of programmed cell death. J. Cell Biol. 124:1–6

Reed, J.C. 1997. Double identity for proteins of the Bcl-2 family. Nature 387:773–776

Schatz, G. 1996. The protein import system of mitochondria. J. Biol. Chem. 271:31763–31766

Schiemann, S., Schwirzke, M., Brunner, N., and Weidle, U.H. 1998. Molecular analysis of two mammary carcinoma cell lines at the transcriptional level as a model system for progression of breast cancer. Clin. Exp. Metast. 16:129–139

Selak, M.A., Armour, S.A., MacKenzie, E.D., Boulahbel, H., Watson, D.G., Mansfield, K.D., Pan, Y., Simon, M.C., Thompson, C.B., and Gottieb, E. 2005. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- prolyl hydroxylase. Cancer Cell 7:77–85

Sies, H. 1999. Free Radic. Biol. Med. 27:916–921

Simons, A.L., Ahmad, I.M., Mattson, D.M., Dornfeld, K.J., Spitz, D.R. 2007. 2-Deoxy-D-glucose combined with cisplatin enhances cytotoxicity via metabolic oxidative stress in human head and neck cancer cells. Cancer Res. 67:3364–3370

Singh, K.K. 1998. Mitochondrial DNA Mutations in Aging, Disease, and Cancer. Springer, New York, USA

Singh, K.K. 2004. Mitochondria damage checkpoint in apoptosis and genome stability. FEMS Yeast Res. 5:127–132

Singh, K.K., Ayyasamy, V., Owens, K., Koul, M.S., Vuciji, M. 2008. Mutations in mitochondrial DNA polymerase gamma associated with breast cancer. Submitted

Singh, K.K., Kulawiec, M., Still, I., Desouki, M.M., Geradts, J., Matsui, S. 2005. Inter-genomic cross talk between mitochondria and the nucleus plays an important role in tumorigenesis. Gene 354:140–146

Singh, K.K., Russell, J., Sigala, B., Zhang, Y., Williams, J., Keshav, K.F. 1999. Mitochondrial DNA determines the cellular response to cancer therapeutic agents. Oncogene 18:6641–6646

Slane, B.G., Aykin-Burns, N., Smith, B., Kalen, A.K., Goswami, P.C., Domann, F.E., and Spitz, D.R. 2006. Mutation of succinate dehydrogenase subunit C results in increased O_2^-, oxidative stress, and genomic instability. Cancer Res. 66:7615–7620

Spelbrink, J.N., Toivonen, J.M., Hakkaart, G.A., Kurkela, J.M., Cooper, H.M., Lehtinen, S.K., Lecrenier, N., Back, J.W., Speijer, D., Foury, F., Jacobs, H.T. 2000. In vivo functional analysis of the human mitochondrial DNA polymerase POLG expressed in cultured human cells. J Biol Chem. 75:24818–24828

Spitz, D.R., Azzam, E.I., Li, J.J., and Gius, D. 2004. Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: A unifiying concept in stress response biology. Cancer Metast. 23:311–322

Sul, H.S., Shrago, E., Goldfarb, S., and Rose, F. 1979. Comparison of the adenine nucleotide translocase in hepatomas and rat liver mitochondria. Biochim. Biophys. Acta 551:148–155

Summerhayes, I.C., Lampidis, T.J., Bernal, S.D., Nadakavukaren, J.J., Nadakavukaren, K.K., Shepherd, E.L., and Chen, L.B. 1982. Unusual retention of rhodamine 123 by mitochondria in muscle and carcinoma cells. Proc. Natl. Acad. Sci. USA 79:5292–5296

Summerhayes, I.C., Wong, D., and Chen, L.B. 1983. Effect of microtubules and intermediate filaments on mitochondrial distribution. J. Cell Sci. 61:87–105

Sun, A.S., and Cederbaum, A.I. 1980. Oxidoreductase activities in normal rat liver, tumor-bearing rat liver, and hepatoma HC-252. Cancer Res. 40:4677–4681

Sun, A.S., Sepkowitz, K., and Geller, S.A. 1981. A study of some mitochondrial and peroxisomal enzymes in human colonic adenocarcinoma. Lab. Invest. 44:13–17

Suzuki, T., Spitz, D.R., Gandhi, P., Lin, H.Y., and Crawford, D.R. 2002. Mammalian resistance to oxidative stress: A comparative analysis. Gene Expression 10:179–191

Taanman, J.W. 1999. The mitochondrial genome: structure, transcription, translation and replication. Biochim. Biophys. Acta. 1410:103–123

Taylor, E.R., Hurrell, F., Shannon, R.J., Lin, T.K., Hirst, J., and Murphy, M.P. 2003. Reversible glutathionylation of complex I increases mitochondrial superoxide formation. J. Biol. Chem. 278:19603–19610

Tomlinson, I.P., Alam, N.A., Rowan, A.J., Barclay, E., Jaeger, E.E., Kelsell, D., Leigh, I., Gorman, P., Lamlum, H., Rahman, S., Roylance, R.R., Olpin, S., Bevan, S., Barker, K., Hearle, N., Houlston, R.S., Kiuru, M., Lehtonen, R., Karhu, A., Vilkki, S., Laiho, P., Eklund, C., Vierimaa, O., Aittomaki, K., Hietala, M., Sistonen, P., Paetau, A., Salovaara, R., Herva, R., Launonen, V., Aaltonen, L.A.; Multiple Leiomyoma Consortium. 2002. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nat. Genetics 30:406–410

Turrens, J.F. 1997. Superoxide production by the mitochondrial respiratory chain. Biosci. Rep. 17:3–8

Turrens, J.F. 2003. Mitochondrial formation of reactive oxygen species. J. Physiol. 522:335–344

Turrens, J.F., and Boveris, A. 1980. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem. J. 191:421–424

Tzagoloff, A. 1982. Mitochondria. Plenum Press, New York, NY, USA

Venturini, I., Zeneroli, M.L., Corsi, L., Avallone, R., Farina, F., Alho, H., Baraldi, C., Ferrarese, C., Pecora, N., Frigo, M., Ardizzone, G., Arrigo, A., Pellici, R., and Baraldi, M. 1998. Up-regulation of peripheral benzodiazepine receptor system in hepatocellular carcinoma. Life Sci. 63:1269–1280

Voet, D., Voet, J.G., and Pratt, C.W. 2002. Fundamentals of Biochemistry. John Wiley & Sons, Inc., New York

Wang, Y., Liu, V.W., Xue, W.C., Tsang, P.C., Cheung, A.N., Ngan, H.Y. 2005. The increase of mitochondrial DNA content in endometrial adenocarcinoma cells: A quantitative study using laser-captured microdissected tissues. Gynecol. Oncol. 98:104–110

Warburg, O. 1930. Metabolism of Tumors. Arnold Constable, London, UK

Warburg, O. 1956. On the origin of cancer cells. Science 123:309–314

Weinhouse, S. 1955. Oxidative metabolism of neoplastic tissue. Adv. Cancer Res. 3:269–325

Weisberg, E.L., Koya, K., Modica-Napolitano, J., Li, Y., Chen, L.B. 1996. In vivo administration of MKT-077 causes partial yet reversible impairment of mitochondrial function. Cancer Res. 56:551–555

Weissig, V., and Torchilin, V.P. 2001. Drug and DNA delivery to mitochondria. Adv. Drug Deliv. Rev. 49:1–2

Wilson, B.C., and Jeeves, W.P. 1987. In Photomedicine, Vol. 2 (Ben-Hur, E., and Rosenthal, I. eds.), pp. 127–177. CRC Press, Boca Raton, FL, USA

Woldegiorgis, G., and Shrago, E. 1985. Adenine nucleotide translocase activity and sensitivity to inhibitors in hepatomas. Comparison of the ADP/ATP carrier in mitochondria and in a purified reconstituted liposome system. J. Biol. Chem. 260:7585–7590

Yen, T.C., King, K.L., Lee, H.C., Yeh, S.H., and Wei, Y.H. 1994. Age-dependent increase of mitochondrial DNA deletions together with lipid peroxides and superoxide dismutase in human liver mitochondria. Free Radic. Biol. Med. 16:207–214

Yakes, F.M., and Van Houten, B. 1997. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc. Natl. Acad. Sci. USA 94:514–519

Zamzami, N. et al. 1996. Mitochondrial control of nuclear apoptosis. J. Exp. Med. 183:1533–1544

Zhang, J., Jin, B., Li, L., Block, E.R., and Patel, J.M. 2005. Nitric oxide-induced persistent inhibition and nitrosylation of active site cysteine residues of mitochondrial cytochrome-c oxidase in lung endothelial cells. Am. J. Physiol. Cell. Physiol. 288:C840–C849

Warburg and his Legacy

Michael Ristow and Tim J. Schulz

Introduction

In the early twentieth century when the German physiologist Otto Warburg forwarded his hypothesis on the origin of cancer cells (Warburg et al. 1924), not much was know on the mechanisms underlying the formation and growth of tumors. Cancer remained a rather mysterious disease, receiving its designation from the ancient Greek physician Hippocrates because of its resemblance to crayfish extremities invading healthy tissue. Warburg, aside from investigating the biochemical fundamentals of cellular respiration and oxygen utilization in the cell, for which he was awarded the 1931 Nobel Prize in physiology and medicine (Warburg 1931), performed hallmark experiments on the metabolic characteristics of tumor cells (Warburg et al. 1924).

However, when Otto Warburg published an updated version of his original articles in the internationally renowned journal *Science* in 1956 (Warburg 1956a), future directions of cancer research appeared to be a matter of fierce debate already (Weinhouse 1956). While the discovery of mutated cancer genes and therapeutics targeting individual types of cancer where subject to relentless criticism by Warburg, a wide body of experimental evidence already existed describing the correlative deterioration of respiration and a concurrent promotion of tumor growth. Warburg concluded "*The irreversible injury of respiration is followed* [...] *by a long struggle for existence by the injured cells to maintain their structure, in which a part of the cells perish from lack of energy, while others succeed by replacing the irretrievably lost respiration energy by fermentation energy*. [...] *Even more harmful in the struggle against cancer can be continual discovery of miscellaneous cancer agents* [...]*, which, by obscuring the underlying phenomena, may hinder necessary preventive measures* [...] (Warburg 1956a)." Consequently, the term "*Warburg effect*" was coined, pinpointing the causal relation of increased production of energy through glycolysis under normoxic conditions at the expense of respiratory oxygen consumption in cancer cells when compared to healthy tissue.

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Fig. 1 Otto Warburg, portrait, date unknown (courtesy of the Max-Planck-Society)

Since then, the general consent on the overall causalities of cancer development has seen some of the most remarkable peripety in modern science. One might even consider Warburg's case as somewhat tragic, one of the centuries leading researchers obsessed with a hypothesis becoming all the more untenable when conflicting insights into cancer biology were reported.

Nowadays, it is generally agreed that cancer evolves following the typical scheme of initiation, promotion, and progression in the lineage of a single cell by accumulating alterations in specific genes, the so-called cancer genes, as the cell divides (Fearon and Vogelstein 1990; Vogelstein and Kinzler 1993). Ever increasing numbers of these cancer genes have been identified, and can preliminarily be subdivided in the two functionally distinct groups of the proto-oncogenes and the tumor suppressor genes (Macdonald and Ford 1992). Although a proto-oncogene acquires its carcinogenic potential through a gain of function, hereby being converted into a full-fledged oncogene, changes in a tumor suppressor gene will only

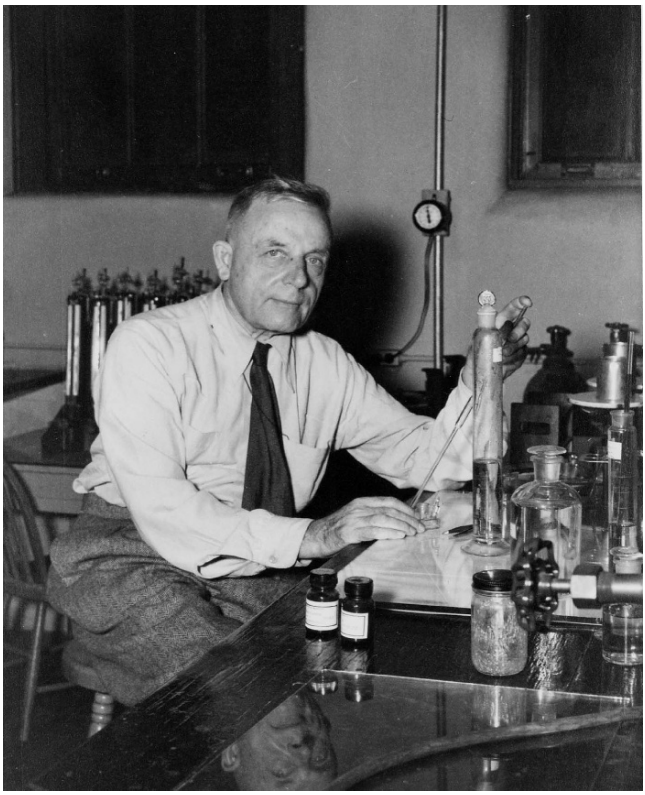

Fig. 2 Otto Warburg in his laboratory, date unknown (courtesy of the Max-Planck-Society)

promote cancer upon loss of normal physiologic function. Both are predominantly achieved through a vast set of different mutations within the DNA, some of which affect single nucleotides only, while others implicate rearrangements of whole chromosomes.

These genes commonly are involved in the control of cell biology processes, including differentiation, proliferation, apoptosis, vascularization, cell migration, and adhesion. All these processes, by different modes of action, are thought to confer growth advantages of a transformed cell over the surrounding tissue and thus bestow abnormal propagation, finally culminating in the formation of a tumor with invasive and metastatic capacities.

Taken together, the two main questions arising from these considerations therefore must be: How can Warburg's hypothesis on the one hand and the nonnegotiable findings on cancer gene biology on the other hand be brought onto a common denominator? And accordingly, to what extent are the known cancer genes, both proto-oncogenes and tumor suppressors, involved in mediation of the Warburg effect?

Genetic Control of Glycolysis Involves Tumor Suppressors and Oncogenes

When comparing energy metabolism of tumors and healthy tissue, profound changes have been observed in literally all cases of cancer or tissue types. Elevated glycolysis has been pinpointed through examinations of the key features of glycolysis in cancer cells on the genomic (Altenberg and Greulich 2004) and, importantly, metabolic level: increased glucose uptake (Birnbaum et al. 1987; Flier et al. 1987), and elevated production of lactic acid as the end-product of glycolysis (Shim et al. 1997).

This observation has been reviewed extensively over the past decades (Pedersen 1978). However, one should be aware that the fundamental principles of energy conversion remain unchanged. Any given cancer cell is able to metabolize a molecule of glucose in the similar way as a normal, untransformed cell within the body would. Glucose is taken up and readily phosphorylated. During the process of glycolysis, two molecules of ATP and pyruvate are formed, the latter then being either oxidized to lactic acid, as the oxygen deprived working muscle cell will do, or decarboxylated by the enzyme pyruvate-dehydrogenase. Shuttled into the mitochondrion as Acetyl-coenzyme A, the stepwise processes of the Krebs cycle further decompose the original compound, eventually yielding CO_2. Energy stored in the chemical bonds of the organic compound is finally completely converted to approximately 36 molecules of ATP per molecule glucose in the respiratory chain (Stryer 1995).

Nonetheless, and as will be discussed in detail later, cancer appears to prefer glycolysis over oxidative phosphorylation. It is important to note that a cancer cell does not lose or gain a principal function. Rather, a metabolic shift takes place while a cancer cell develops, probably slow in its progression, away from the mitochondrial energy production towards increased reliance onto anoxic breakdown of substrate, such as glucose. In healthy, i.e., normal cells, oxygen controls the ratio of glycolysis towards mitochondrial respiration, an effect that has long since been known as the Pasteur effect (Pasteur 1861). By allowing high rates of glycolysis also in the presence of oxygen, with lactic acid as the ultimate end-product, the Warburg effect seems diametrically opposed to the findings of Pasteur. However, numerous reports accumulate to date indicating that exactly this is the case, tentatively suggesting that the metabolic shift might be a key mechanism to support the fabled "growth advantage" a cancer cell needs to overgrow its contemporaries.

In modern medicine, a technique called positron emission tomography (PET) is of outstanding importance in detecting tumor tissue. By employing the glucose analogue ^{18}-Fluoro-deoxy-D-glucose as a tracer substance during PET analysis, tissues with extraordinary high uptake of glucose can easily be identified, hereby not only facilitating localization and prognosis in cancer patients but also indicating that metabolic adaptations of cancer cells leading to increased glycolysis play a key role in tumor biology (Hawkins and Phelps 1988; Gambhir 2002).

In an insightful review, Gatenby and Gillies propose an evolutionary model to explain the development of the apparently high glycolytic rates of cancer cells

(Gatenby and Gillies 2004): Under conditions of repeated or prolonged hypoxia, which may occur owing to hyperplastic growth, selection is in favor of cells with increased glycolysis (Raghunand et al. 2003). Additionally, increased glycolytic flux leads to increased acidification owing to elevated production of lactate. Again, selection is in favor of cells that have increased resistance towards the disadvantageous acidic microenvironment. Meanwhile, this shift assumes the abandonment of maximum energetic yield per molecule of substrate, i.e. from 36 molecules of ATP to merely 2 molecules of glycolytic ATP. Therefore, a pre-malignant lesion will be less dependent on oxygen and is able to proliferate under conditions where a healthy cell would be growth-inhibited. In support of the Warburg hypothesis, this model provides a potential explanation on why cancer cells benefit from an energetic tradeoff although at first glance, it appears to result in an adverse physiological condition.

Several experimental approaches further emphasize this view: Upregulation of glucose transporters (GLUTs), specifically GLUT1, has repeatedly been correlated with tumor aggressiveness and poor diagnosis (Younes et al. 1996; Grover-McKay et al. 1998; Sakashita et al. 2001). Importantly, experimental evidence links several well established cancer genes to control of the glycolytic rate. Specifically, it has been demonstrated that the *c-myc* oncogene upregulates the expression of *GLUT1* and several genes involved in glycolysis, namely glucose-6-phosphate isomerase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and enolase (Osthus et al. 2000). Of note, c-Myc also functions to transactivate the lactate dehydrogenase A (*LDHA*) gene, which is necessary for c-Myc-mediated transformation in fibroblasts (Shim et al. 1997). Concurrently, expression of the ras- and src-oncogenes lead to increased glucose import into the resulting transformed cells (Flier et al. 1987). Furthermore, and as c-Myc itself, they were able to coactivate *LDHA* gene expression via binding to a carbohydrate responsive element located in the *LDHA* promoter region (Dang et al. 1997), altogether supporting the metabolic shift as stated by the Warburg effect.

One of the key mechanisms to control glycolysis and metabolic adaptation to conditions of low oxygen supply is the hypoxia induced factor 1 alpha (HIF1α). After its initial identification in 1992 (Semenza and Wang 1992), a large body of evidence has accumulated, suggesting that HIF1α might in fact be responsible for many of the effects as proposed in Warburg's hypothesis (Robey et al. 2005). Briefly, HIF1α forms heterodimers with its partner HIF1β to yield the active transcription factor which binds to promoter elements of numerous genes encoding for proteins of glucose transport (Ebert et al. 1995) and activating the expression of most enzymes of the glycolytic pathway including *LDHA* (Semenza et al. 1994; Firth et al. 1995). Furthermore, the active HIF-dimer induces the expression of growth factors mediating enhanced angiogenesis and erythropoesis (Semenza and Wang 1992; Maxwell et al. 1997). HIF1α is expressed constitutively in healthy tissues, and rapidly degraded after ubiquitinylation under normoxic conditions, i.e., conditions of normal oxygen partial pressure on the cellular level. However, when oxygen supply is low due to impaired blood flow or inadequate vascularization, HIF1α degradation is inhibited, leading to increased transcription factor activity

allowing tight regulation of the respective target genes owing to minute changes in oxygenation (Semenza 2001). Notably, deregulated HIF1α or sustained stability under normal oxygen supply has been linked to numerous types of cancer in the past years since its discovery (Semenza 2007). This is further underlined by the fact, that the responsible ubiquitin ligase, the so-called von Hippel–Lindau tumor suppressor (VHL) is the ultimate cause of the von Hippel–Lindau syndrome, which is associated with increased incidence of retinal, neuronal, and renal neoplasias (McKusick et al. 2007). Aside from hypoxia caused by inadequate vascularization, also downregulation of pyruvate dehydrogenase might lead to accumulation of pyruvate and increased lactic acid production, which contributes to stabilization of HIF1α (Koukourakis et al. 2005). This has been confirmed in a study showing activation and stabilization of HIF1α by means of altered levels of pyruvate and lactate in the cell (Lu et al. 2002). Hence, although hypoxia might play a key role in tumor development, it seems not to be unambiguously required for sustained increases in glycolysis. Possibly, this might even contribute to a vicious cycle of enhanced glycolysis leading to increased cellular accumulation of pyruvate and lactate, which in turn stimulate HIF1α and thereby promote further increases in expression of glycolytic enzymes (Lu et al. 2005).

As another potential candidate pathway regulating the glycolytic phenotype of cancer cells, the Akt/PBK (protein kinase B) survival pathway has been described. Akt/PKB has previously been known for its ability to induce glucose uptake and glycolysis independently of malignant transformation (Rathmell et al. 2003). In cancer cells, increased glycolysis was associated with activation of Akt/PKB (Elstrom et al. 2004). Accordingly, glioblastoma cell lines lacking constitutive activation of Akt/PKB did not display increased rates of glycolysis, whereas expression of Akt/PBK accelerated cell death under glucose deprived culture conditions, altogether suggesting that deregulated Akt/PKB signaling might constitute an alternative mechanism to exert the Warburg effect in cancer.

Lastly, interaction of enzymes of the glycolytic pathway with the p53 tumor suppressor protein have been observed further underscoring the relevance of cancer genes in facilitating a metabolic shift towards increased glycolysis. In this context, the prominent role of hexokinase in the glycolytic activity of cancer cells has been described. Interestingly, in hepatic cancer cells, hexokinase was closely associated to the mitochondria, which was not the case in healthy liver cells (Bustamante and Pedersen 1977; Bustamante et al. 1981). A later study revealed that mutations in the p53 tumor suppressor protein might activate hexokinase II expression through binding at distinct sites of the hexokinase promoter (Mathupala et al. 1997). Also, it has been reported that loss of p53 not only increased glycolytic activity but specifically led to increased hexokinase activity in a breast cancer cell line (Smith et al. 2006). In line with these findings, the protein TIGAR (TP53-induced glycolysis and apoptosis regulator), a p53 inducible isoform of phosphofructo-kinase, has recently been identified (Bensaad et al. 2006). TIGAR functions as a regulator of glycolysis, exerting an inhibition of glycolysis by lowering levels of fructose-2,6-bisphosphate. Likewise, when *p53* is mutated in cancer, TIGAR activity is lowered resulting in increased glycolysis.

Taken together, numerous cancer genes have been demonstrated to promote the typical metabolic changes characteristic of the Warburg effect.

Cancer Genes Impose Reduced Mitochondrial Activity

Although control mechanisms regulating glycolysis, as discussed so far, appear to be of major importance in exacerbating the Warburg effect, many aspects of mitochondrial physiology further underscore that oxidative metabolism is also affected in cancer cells, thus representing the other side of the coin. In a recent review, Herrmann et al. discuss the observation that complexes of the mitochondrial respiratory chain, such as cytochrome c oxidase (COX), have diminished activity in cancer, mainly due to decreased expression of the subunits encoded by the mitochondrial DNA (Herrmann and Herrmann 2007). Concurrently, mutations in the mitochondrial DNA have repeatedly been associated with tumorigenesis (Baggetto 1992; Carew and Huang 2002; Singh 2004; Petros et al. 2005; Taylor and Turnbull 2005). The phenomenon of deteriorated mitochondrial activity appears to be of a broad distribution in numerous cancer specimens (Isidoro et al. 2004; Hervouet et al. 2005; Wu et al. 2007) and alterations on the proteome level have been proposed as biomarkers for clinical diagnosis of cancer (Isidoro et al. 2005; Cuezva et al. 2004). Hence, alterations in mitochondrial energetic physiology might contribute to the Warburg effect in that impaired mitochondrial oxidative phosphorylation parallels upregulation of glycolysis. For instance, in tumor samples derived from different tissue types, it could be demonstrated that either a general reduction in mitochondrial mass or specifically reduced expression of ATP synthase subunits is associated with cancerous tissue, further supporting the notion that a defect in mitochondrial bioenergetics might be a specific characteristic during carcinogenesis (Cuezva et al. 2002).

In line with these findings, it has been demonstrated that the tumor suppressor protein p53 might be directly involved in regulating mitochondrial respiration through regulation of COX activity (Zhou et al. 2003). Colon cell lines lacking p53 furthermore displayed decreased rates of ATP synthesis via oxidative phosphorylation and decreased overall respiratory activity, while sustained growth rates and ATP levels could be mainly attributable to a concurrent increase in glycolysis (Matoba et al. 2006; Ma et al. 2007). While this regulation was dependent on the presence of SCO2 (synthesis of cytochrome c oxidase 2), which is involved in assembly of the mature complex III of the respiratory chain (Matoba et al. 2006) it remains to be shown whether this pathway has any effect on mitochondrial activity. Hence, p53 not only acts by regulating glycolysis as discussed above but theoretically also directly affects respiration, hereby providing an hypothetical link between altered energy metabolism and the pleiotropic roles of p53 in tumor development as of one of the most prominent cancer genes known today.

Along with their capability to promote increased glycolysis, components of either the HIF1α or Akt/PKB signaling pathway appear to be involved in regulation

of mitochondrial activity. HIF1α has been demonstrated to activate pyuvate dehydrogenase kinase (PDK), which in turn inactivates pyruvate dehydrogenase via phosphorylation, preventing the decarboxylation of pyruvate to AcCoA and entry of this metabolite into the mitochondrion. In this context, it could be observed that HIF1α directly regulated the expression of PDK by genetic transactivation in response to oxygen availability and by this means had a direct negative impact on mitochondrial oxygen consumption (Papandreou et al. 2006; Kim et al. 2006). Tumor suppressor protein VHL is not only involved in ubiquitinylation of HIF1α but also exerts positive effects on the expression of markers of mitochondrial biogenesis and activity. Loss of VHL, as observed in different types cancer and in analogy to Warburg's hypothesis, might therefore contribute to defective mitochondrial function (Hervouet et al. 2005). Regarding Akt/PKB signaling, inactivation of mitochondrial oxidative phosphorylation by various means has been shown to increase phosphorylation of the oncogene Akt/PKB (protein kinase B) (Pelicano et al. 2006a, b). Mechanistically, the authors propose enhanced accumulation of NADH has been proposed, which in turn leads to inactivation of the tumor suppressor PTEN (Stiles et al. 2004), which under normal conditions inhibits the Akt/PKB survival pathway (Pelicano et al. 2006a, b). As mutations in the mitochondrial DNA have repeatedly been reported in numerous cancer tissues, defective oxidative phosphorylation might contribute to an Akt/PKB-mediated energetic shift. Corresponding to the Warburg effect this could explain the enigma of preference of inefficient glycolytic energy conversion which nonetheless allows continued growth and increased resistance to hypoxic stress and apoptosis.

When considering impaired mitochondrial function as a key property of malignant transformation, activation of the mitochondrial energy conversion by different means might oppose the Warburg effect, rendering cancer cells more prone to therapeutic intervention or even reducing malignancy per se. Accordingly, it could be demonstrated that activation of mitochondrial energy conversion by forced expression of the mitochondrial protein frataxin leads to reduced growth of cancer cells *in vitro* and *in vivo* (Schulz et al. 2006). Frataxin, encoded in the nucleus, is involved in iron–sulfur (Fe/S) cluster biogenesis. These structures are of major importance for the functionality of enzymes of the mitochondrial energy conversion (Mühlenhoff et al. 2002; Gerber et al. 2003). As a possible signaling pathway relaying increased mitochondrial metabolism and growth, the mitogen activated protein kinase p38 has been identified. P38 has previously been discussed as a potential tumor suppressor protein (Bulavin and Fornace 2004; Timofeev et al. 2005). Accordingly, liver-specific inactivation of frataxin in the mouse specifically impaired the activity of Fe/S containing enzymes of the Krebs cycle, i.e. aconitase, and the respiratory chain, leading to increased incidence of hepatic adenomas (Thierbach et al. 2005). In line with these findings, defective Krebs cycle action has been observed in colorectal carcinoma tissue samples (Bi et al. 2006). Of note, also aconitase expression was found to be impaired in this study. The authors suggest that impaired Krebs cycle activity in conjunction with enhanced glycolysis functions to stabilize HIF1α to facilitate the Warburg effect in colorectal cancer. These findings taken together suggest that a general activation of the mitochondrial

energy metabolism may act in opposition to the Warburg effect and could be a valuable tool in cancer therapy.

One of the most recently identified pathways involved in control of energy metabolism comprises the signaling events centering on the AMP activated protein kinase (AMPK). To date, AMPK has been firmly established as a key controller of bioenergetics actively regulating a multitude of biochemical reactions that result in increased cellular ATP content (Kahn et al. 2005). AMPK is activated in conditions of low energy supply, as a consequence of stressors like hypoxia or reduced nutrient access. Under these conditions, cellular AMP content rises and functions as a direct allosteric activator of AMPK. For complete activation of AMPK, however, phosphorylation is required, which is mainly accomplished by the upstream tumor suppressor kinase, LKB1 (Alessi et al. 2006). Mutation of *LKB1* has been identified as the causal factor in Peutz-Jeghers syndrome, which is characterized by increased incidence of cancer in different tissues. As important downstream targets of AMPK, two other tumor suppressors which are mutated in tuberous sclerosis, TSC1 and 2, play a key role in protein synthesis (Inoki et al. 2003). One of the major outcomes of AMPK and activation of its downstream effectors is induction of mitochondrial biogenesis and activity (Reznick and Shulman 2006). With respect to cancer biology, AMPK is involved in a complex network controlling numerous physiological processes such as protein synthesis and proliferation and has been reviewed extensively in the recent literature (Shaw 2006). AMPK has been demonstrated to functionally interact with some of the cancer genes that have been discussed so far (Shaw 2006). Of special interest in this context, AMPK has been shown to activate the p53 tumor suppressor leading to cell cycle arrest or premature cellular senescence upon prolonged activation when glucose availability is restricted (Jones et al. 2005). Given the presence of a number of tumor suppressors that are linked to AMPK, such as TSC and LKB1, and the fact that AMPK itself is of paramount importance to mitochondrial bioenergetics, downregulation of this signaling pathway might be of potential relevance in promoting the Warburg effect in cancer.

The Warburg Effect as Potential Therapeutic Target

The Warburg effect, as it was described more than 80 years ago, still represents a matter of extensive debate on whether or not it is a primary cause or merely a consequence of tumorigenesis. Although recent data strongly suggest that mutations in certain cancer genes very well might contribute to the metabolic shift of the Warburg effect and result in improved fitness of the resulting cells, this is of a somewhat subordinate interest for therapeutic strategies to deal with cancer. In recent years, a group of potential cancer therapeutics has been described targeting the inherent metabolic properties of the Warburg effect (Pelicano et al. 2006a). In glioblastoma cells known to rely on glycolysis, withdrawal of the major energy source, glucose, induced apoptosis whereas normal astrocytes displayed a shift of energy utilization towards other sources such as fatty acids (Jelluma et al. 2006).

Accordingly, agents that inhibit glycolysis have been proposed as possible co-therapeutics to established cancer therapies which affect the inherent characteristics of cancer cell metabolism (Maschek et al. 2004). Some of these agents, such as 2-deoxy-D-glucose (2DG) and lonidamine, have notably reached the level of clinical trials already (De Lena et al. 2001; Di Cosimo et al. 2003; Oudard et al. 2003; Singh et al. 2005).

2DG has long since been known for its ability to inhibit glycolysis (Sols and Crane 1954; Brown 1962). Its growth inhibitory action in cancer cells has been documented in a number of experimental settings *in vitro* and *in vivo* (Zhu et al. 2005; Zhang et al. 2006). However, it should be noted that 2DG at higher doses leads to increased incidents of congestive heart failure in rats (Lane 1998; Ingram et al. 2006), somewhat dampening the high promises of this compound and its applicability in human cancer therapy. As with 2DG, 3-bromopyruvate (3BrPA) inhibits glycolysis. A lactic acid analog, 3BrPA has been identified as a compound preferentially targeting cancer cells. The 3BrPA inhibition target is hexokinase, causing massive depletion of cellular ATP concentrations and induction of apoptosis (Xu et al. 2005). Furthermore, experimental evidence confirms the properties of 3BrPA as a highly effective antitumor compound in vivo in different animal models and different types of cancer (Ko et al. 2001; Geschwind et al. 2002; Ko et al. 2004). Likewise, a number of glucose-analogous compounds may provide potential agents which require further inverstigation, such as 5-thioglucose and mannoheptulose (Chen et al. 2007).

Indazole-3-carboylic acid related compounds, such as lonidamine, display inhibitory action on the site of glycolysis (Floridi et al. 1981). Lactic acid production has been shown to decrease after administration of lonidamine, intriguingly circumventing the Warburg effect in a case study of leukemia (Natali et al. 1984). Later, it has been demonstrated that lonidamine appears to be an overall inhibitor of energy metabolism, decreasing both glycolysis and respiration dose-dependently (Floridi et al. 1998). Nonetheless, and as stated above, lonidamine has found its way into a number of promising clinical trials. Consistent with this, it has been observed that downregulation of LDHA might exert positive effect in cancer therapy. Downregulation of LDHA expression led to increased respiratory activity and reduced malignancy in vitro (Fantin et al. 2006). Accordingly, metabolic inhibitors of LDHA, for instance oxamate, might yield highly relevant therapeutic approaches (Chen et al. 2007).

Summary

It has been suggested that the Warburg effect on one side (Warburg et al. 1926, 1930, 1956) and mutated cancer genes on the other side (Fearon and Vogelstein 1990; Vogelstein and Kinzler 1993) might not be mutually exclusive at all (Ramanathan et al. 2005). Rather, there appears to be a close link between these two cancer theories in that alterations of the genetic information, namely in well

established oncogenes and tumor suppressor genes, might exert metabolic consequences leading to the Warburg effect. Naturally, a precedent change in the genetic repertoire of a cell is peremptory to changes in cellular physiology, a fact that Otto Warburg could not possibly have been aware of in the early decades of the last century. Nonetheless, Theodore Boveri, another leading physiologist of the beginning of 20th century, had concluded from his own hallmark work on sea urchin embryos that chromosomal rearrangements could be held accountable for the unrestricted growth characteristics of cancer cells (Boveri 1902, 1914; Manchester 1997). A genetic function will only entail consequences such as malignant growth when it affects the overall phenotype of a given cell, for instance its metabolic properties. A vast body of experimental evidence introduces numerous pleiotropic effects of single cancer genes, which ultimately can cause the disease to evolve from a healthy cell. Once again, evidence emerges that the Warburg effect might actually constitute more than an anecdotal by-effect of cancerogenesis. As has here been discussed, a set of different signaling pathways alone or in conjunction with each other may function to promote the glycolytic phenotype of tumors and in parallel decrease mitochondrial activity, resulting in what could also be named the Warburg phenotype of a cell. Namely these pathways include HIF1α signaling, the Akt/PBK cascade, the p53 tumor suppressor, and controllers of energy metabolism such as p38 and AMPK. All these cooperate in the healthy cell to maintain physiologic homeostasis. And, as likely, this may result in significant phenotypic changes once deregulation of this intricate network occurs.

Finally, two conclusions can be drawn from our current state of knowledge on cancer biology: First, the Warburg effect is an inherent property of cancers, a direct consequence of altered genetic information that promotes the growth of tumors; second, pharmacologically counteracting the Warburg effect may provide novel therapeutic concepts ultimately leading to improved public health.

References

Alessi, D. R., Sakamoto, K., and Bayascas, J. R. 2006. LKB1-dependent signaling pathways. Annu Rev Biochem, *75:* 137–163.

Altenberg, B. and Greulich, K. O. 2004. Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. Genomics, *84:* 1014–1020.

Baggetto, L. G. 1992. Role of mitochondria in carcinogenesis. Eur J Cancer, *29A:* 156–159.

Bensaad, K., Tsuruta, A., Selak, M. A., Vidal, M. N., Nakano, K., Bartrons, R., Gottlieb, E., and Vousden, K. H. 2006. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell, *126:* 107–120.

Bi, X., Lin, Q., Foo, T. W., Joshi, S., You, T., Shen, H. M., Ong, C. N., Cheah, P. Y., Eu, K. W., and Hew, C. L. 2006. Proteomic analysis of colorectal cancer reveals alterations in metabolic pathways: mechanism of tumorigenesis. Mol Cell Proteomics, *5:* 1119–1130.

Birnbaum, M. J., Haspel, H. C., and Rosen, O. M. 1987. Transformation of rat fibroblasts by FSV rapidly increases glucose transporter gene transcription. Science, *235:* 1495–1498.

Boveri, T. 1902. Über mehrpolige Mitosen als Mittel zur Analyse des Zellkerns., Vol. 35, p. 67–90. Wurzburg: Vehr. d. phys. med. Ges. zu Wurzburg.

Boveri, T. 1914. Zur Frage der Entstehung maligner Tumoren. Jena: Gustav Fischer Verlag.
Brown, J. 1962. Effects of 2-deoxyglucose on carbohydrate metablism: review of the literature and studies in the rat. Metabolism, *11:* 1098–1112.
Bulavin, D. V. and Fornace, A. J., Jr. 2004. p38 MAP kinase's emerging role as a tumor suppressor. Adv Cancer Res, *92:* 95–118.
Bustamante, E. and Pedersen, P. L. 1977. High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase. Proc Natl Acad Sci USA, *74:* 3735–3739.
Bustamante, E., Morris, H. P., and Pedersen, P. L. 1981. Energy metabolism of tumor cells. Requirement for a form of hexokinase with a propensity for mitochondrial binding. J Biol Chem, *256:* 8699–8704.
Carew, J. S. and Huang, P. 2002. Mitochondrial defects in cancer. Mol Cancer, *1:* 9.
Chen, Z., Lu, W., Garcia-Prieto, C., and Huang, P. 2007. The Warburg effect and its cancer therapeutic implications. J Bioenerg Biomembr, *39(3)*: 267–274.
Cuezva, J. M., Krajewska, M., de Heredia, M. L., Krajewski, S., Santamaria, G., Kim, H., Zapata, J. M., Marusawa, H., Chamorro, M., and Reed, J. C. 2002. The bioenergetic signature of cancer: a marker of tumor progression. Cancer Res, *62:* 6674–6681.
Cuezva, J. M., Chen, G., Alonso, A. M., Isidoro, A., Misek, D. E., Hanash, S. M., and Beer, D. G. 2004. The bioenergetic signature of lung adenocarcinomas is a molecular marker of cancer diagnosis and prognosis. Carcinogenesis, *25:* 1157–1163.
Dang, C. V., Lewis, B. C., Dolde, C., Dang, G., and Shim, H. 1997. Oncogenes in tumor metabolism, tumorigenesis, and apoptosis. J Bioenerg Biomembr, *29:* 345–354.
De Lena, M., Lorusso, V., Latorre, A., Fanizza, G., Gargano, G., Caporusso, L., Guida, M., Catino, A., Crucitta, E., Sambiasi, D., and Mazzei, A. 2001. Paclitaxel, cisplatin and lonidamine in advanced ovarian cancer. A phase II study. Eur J Cancer, *37:* 364–368.
Di Cosimo, S., Ferretti, G., Papaldo, P., Carlini, P., Fabi, A., and Cognetti, F. 2003. Lonidamine: efficacy and safety in clinical trials for the treatment of solid tumors. Drugs Today (Barc), *39:* 157–174.
Ebert, B. L., Firth, J. D., and Ratcliffe, P. J. 1995. Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. J Biol Chem, *270:* 29083–29089.
Elstrom, R. L., Bauer, D. E., Buzzai, M., Karnauskas, R., Harris, M. H., Plas, D. R., Zhuang, H., Cinalli, R. M., Alavi, A., Rudin, C. M., and Thompson, C. B. 2004. Akt stimulates aerobic glycolysis in cancer cells. Cancer Res, *64:* 3892–3899.
Fantin, V. R., St-Pierre, J., and Leder, P. 2006. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell, *9:* 425–434.
Fearon, E. R. and Vogelstein, B. 1990. A genetic model for colorectal tumorigenesis. Cell, *61:* 759–767.
Firth, J. D., Ebert, B. L., and Ratcliffe, P. J. 1995. Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. J Biol Chem, *270:* 21021–21027.
Flier, J. S., Mueckler, M. M., Usher, P., and Lodish, H. F. 1987. Elevated levels of glucose transport and transporter messenger RNA are induced by ras or src oncogenes. Science, *235:* 1492–1495.
Floridi, A., Paggi, M. G., Marcante, M. L., Silvestrini, B., Caputo, A., and De Martino, C. 1981. Lonidamine, a selective inhibitor of aerobic glycolysis of murine tumor cells. J Natl Cancer Inst, *66:* 497–499.
Floridi, A., Bruno, T., Miccadei, S., Fanciulli, M., Federico, A., and Paggi, M. G. 1998. Enhancement of doxorubicin content by the antitumor drug lonidamine in resistant Ehrlich ascites tumor cells through modulation of energy metabolism. Biochem Pharmacol, *56:* 841–849.
Gambhir, S. S. 2002. Molecular imaging of cancer with positron emission tomography. Nat Rev Cancer, *2:* 683–693.

Gatenby, R. A. and Gillies, R. J. 2004. Why do cancers have high aerobic glycolysis? Nat Rev Cancer, *4:* 891–899.

Gerber, J., Mühlenhoff, U., and Lill, R. 2003. An interaction between frataxin and Isu1/Nfs1 that is crucial for Fe/S cluster synthesis on Isu1. EMBO Rep, *4:* 906–911.

Geschwind, J. F., Ko, Y. H., Torbenson, M. S., Magee, C., and Pedersen, P. L. 2002. Novel therapy for liver cancer: direct intraarterial injection of a potent inhibitor of ATP production. Cancer Res, *62:* 3909–3913.

Grover-McKay, M., Walsh, S. A., Seftor, E. A., Thomas, P. A., and Hendrix, M. J. 1998. Role for glucose transporter 1 protein in human breast cancer. Pathol Oncol Res, *4:* 115–120.

Hawkins, R. A. and Phelps, M. E. 1988. PET in clinical oncology. Cancer Metastasis Rev, *7:* 119–142.

Herrmann, P. C. and Herrmann, E. C. 2007. Oxygen metabolism and a potential role for cytochrome c oxidase in the Warburg effect. J Bioenerg Biomembr, *39(3)*: 247–250.

Hervouet, E., Demont, J., Pecina, P., Vojtiskova, A., Houstek, J., Simonnet, H., and Godinot, C. 2005. A new role for the von Hippel-Lindau tumor suppressor protein: stimulation of mitochondrial oxidative phosphorylation complex biogenesis. Carcinogenesis, *26:* 531–539.

Ingram, D. K., Zhu, M., Mamczarz, J., Zou, S., Lane, M. A., Roth, G. S., and deCabo, R. 2006. Calorie restriction mimetics: an emerging research field. Aging Cell, *5:* 97–108.

Inoki, K., Zhu, T., and Guan, K. L. 2003. TSC2 mediates cellular energy response to control cell growth and survival. Cell, *115:* 577–590.

Isidoro, A., Martinez, M., Fernandez, P. L., Ortega, A. D., Santamaria, G., Chamorro, M., Reed, J. C., and Cuezva, J. M. 2004. Alteration of the bioenergetic phenotype of mitochondria is a hallmark of breast, gastric, lung and oesophageal cancer. Biochem J, *378:* 17–20.

Isidoro, A., Casado, E., Redondo, A., Acebo, P., Espinosa, E., Alonso, A. M., Cejas, P., Hardisson, D., Fresno Vara, J. A., Belda-Iniesta, C., Gonzalez-Baron, M., and Cuezva, J. M. 2005. Breast carcinomas fulfill the Warburg hypothesis and provide metabolic markers of cancer prognosis. Carcinogenesis, *26:* 2095–2104.

Jelluma, N., Yang, X., Stokoe, D., Evan, G. I., Dansen, T. B., and Haas-Kogan, D. A. 2006. Glucose withdrawal induces oxidative stress followed by apoptosis in glioblastoma cells but not in normal human astrocytes. Mol Cancer Res, *4:* 319–330.

Jones, R. G., Plas, D. R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M. J., and Thompson, C. B. 2005. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol Cell, *18:* 283–293.

Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. 2005. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab, *1:* 15–25.

Kim, J. W., Tchernyshyov, I., Semenza, G. L., and Dang, C. V. 2006. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab, *3:* 177–185.

Ko, Y. H., Pedersen, P. L., and Geschwind, J. F. 2001. Glucose catabolism in the rabbit VX2 tumor model for liver cancer: characterization and targeting hexokinase. Cancer Lett, *173:* 83–91.

Ko, Y. H., Smith, B. L., Wang, Y., Pomper, M. G., Rini, D. A., Torbenson, M. S., Hullihen, J., and Pedersen, P. L. 2004. Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. Biochem Biophys Res Commun, *324:* 269–275.

Koukourakis, M. I., Giatromanolaki, A., Sivridis, E., Gatter, K. C., and Harris, A. L. 2005. Pyruvate dehydrogenase and pyruvate dehydrogenase kinase expression in non small cell lung cancer and tumor-associated stroma. Neoplasia, *7:* 1–6.

Lane, M. A. 1998. 2-Deoxy-D-Glucose Feeding in Rats Mimics Physiologic Effects of Calorie Restriction. JOURNAL OF ANTI-AGING MEDICINE, *1:* 327–336.

Lu, H., Forbes, R. A., and Verma, A. 2002. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem, *277:* 23111–23115.

Lu, H., Dalgard, C. L., Mohyeldin, A., McFate, T., Tait, A. S., and Verma, A. 2005. Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. J Biol Chem, *280:* 41928–41939.

Ma, W., Sung, H. J., Park, J. Y., Matoba, S., and Hwang, P. M. 2007. A pivotal role for p53: balancing aerobic respiration and glycolysis. J Bioenerg Biomembr, *39(3)*: 243–246.

Macdonald, F. and Ford, C. H. J. 1992. Oncogenes and tumor Suppressor genes (Medical perspectives series), p. 112. Oxford: BIOS Scientfic Publishers.

Manchester, K. 1997. The quest by three giants of science for an understanding of cancer. Endeavour, *21:* 72–76.

Maschek, G., Savaraj, N., Priebe, W., Braunschweiger, P., Hamilton, K., Tidmarsh, G. F., De Young, L. R., and Lampidis, T. J. 2004. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. Cancer Res, *64:* 31–34.

Mathupala, S. P., Heese, C., and Pedersen, P. L. 1997. Glucose catabolism in cancer cells. The type II hexokinase promoter contains functionally active response elements for the tumor suppressor p53. J Biol Chem, *272:* 22776–22780.

Matoba, S., Kang, J. G., Patino, W. D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P. J., Bunz, F., and Hwang, P. M. 2006. p53 regulates mitochondrial respiration. Science, *312:* 1650–1653.

Maxwell, P. H., Dachs, G. U., Gleadle, J. M., Nicholls, L. G., Harris, A. L., Stratford, I. J., Hankinson, O., Pugh, C. W., and Ratcliffe, P. J. 1997. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci USA, *94:* 8104–8109.

McKusick, V. A., Kniffin, C. L., Tiller, G. E., Wright, M. J., Hamosh, A., Antonarakis, S. E., Rasmussen, S. A., Smith, M., Brennan, P., and Rasooly, R. S. 2007. Online mendelian inheritance in man: von Hippel-Lindau syndrome (OMIM 193300). http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=193300.

Mühlenhoff, U., Richhardt, N., Ristow, M., Kispal, G., and Lill, R. 2002. The yeast frataxin homolog Yfh1p plays a specific role in the maturation of cellular Fe/S proteins. Hum Mol Genet, *11:* 2025–2036.

Natali, P. G., Salsano, F., Viora, M., Nista, A., Malorni, W., Marolla, A., and De Martino, C. 1984. Inhibition of aerobic glycolysis in normal and neoplastic lymphoid cells induced by Lonidamine [1-(2,4-dichlorobenzyl)-I-H-indazol-3-carboxylic acid]. Oncology, *41* (Suppl. 1)*:* 7–14.

Osthus, R. C., Shim, H., Kim, S., Li, Q., Reddy, R., Mukherjee, M., Xu, Y., Wonsey, D., Lee, L. A., and Dang, C. V. 2000. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem, *275:* 21797–21800.

Oudard, S., Carpentier, A., Banu, E., Fauchon, F., Celerier, D., Poupon, M. F., Dutrillaux, B., Andrieu, J. M., and Delattre, J. Y. 2003. Phase II study of lonidamine and diazepam in the treatment of recurrent glioblastoma multiforme. J Neurooncol, *63:* 81–86.

Papandreou, I., Cairns, R. A., Fontana, L., Lim, A. L., and Denko, N. C. 2006. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab, *3:* 187–197.

Pasteur, L. 1861. Influence de l'oxygene sur le developpement de la levure et la fermentation alcoolique. Bulletin de la Societe Chimique de Paris, p. 79–80.

Pedersen, P. L. 1978. Tumor mitochondria and the bioenergetics of cancer cells. Prog Exp Tumor Res, *22:* 190–274.

Pelicano, H., Martin, D. S., Xu, R. H., and Huang, P. 2006a. Glycolysis inhibition for anticancer treatment. Oncogene, *25:* 4633–4646.

Pelicano, H., Xu, R. H., Du, M., Feng, L., Sasaki, R., Carew, J. S., Hu, Y., Ramdas, L., Hu, L., Keating, M. J., Zhang, W., Plunkett, W., and Huang, P. 2006b. Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox-mediated mechanism. J Cell Biol, *175:* 913–923.

Petros, J. A., Baumann, A. K., Ruiz-Pesini, E., Amin, M. B., Sun, C. Q., Hall, J., Lim, S., Issa, M. M., Flanders, W. D., Hosseini, S. H., Marshall, F. F., and Wallace, D. C. 2005. mtDNA mutations increase tumorigenicity in prostate cancer. Proc Natl Acad Sci USA, *102:* 719–724.

Raghunand, N., Gatenby, R. A., and Gillies, R. J. 2003. Microenvironmental and cellular consequences of altered blood flow in tumours. Br J Radiol, *76* (Spec No. 1): S11-S22.

Ramanathan, A., Wang, C., and Schreiber, S. L. 2005. Perturbational profiling of a cell-line model of tumorigenesis by using metabolic measurements. Proc Natl Acad Sci USA, *102:* 5992–5997.

Rathmell, J. C., Fox, C. J., Plas, D. R., Hammerman, P. S., Cinalli, R. M., and Thompson, C. B. 2003. Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. Mol Cell Biol, *23:* 7315–7328.

Reznick, R. M. and Shulman, G. I. 2006. The role of AMP-activated protein kinase in mitochondrial biogenesis. J Physiol, *574:* 33–39.

Robey, I. F., Lien, A. D., Welsh, S. J., Baggett, B. K., and Gillies, R. J. 2005. Hypoxia-inducible factor-1alpha and the glycolytic phenotype in tumors. Neoplasia, *7:* 324–330.

Sakashita, M., Aoyama, N., Minami, R., Maekawa, S., Kuroda, K., Shirasaka, D., Ichihara, T., Kuroda, Y., Maeda, S., and Kasuga, M. 2001. Glut1 expression in T1 and T2 stage colorectal carcinomas: its relationship to clinicopathological features. Eur J Cancer, *37:* 204–209.

Schulz, T. J., Thierbach, R., Voigt, A., Drewes, G., Mietzner, B. H., Steinberg, P., Pfeiffer, A. F., and Ristow, M. 2006. Induction of oxidative metabolism by mitochondrial frataxin inhibits cancer growth: Otto Warburg revisited. J Biol Chem, *281:* 977–981.

Semenza, G. L. 2001. HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. Cell, *107:* 1–3.

Semenza, G. L. 2007. HIF-1 mediates the Warburg effect in clear cell renal carcinoma. J Bioenerg Biomembr, *39(3)*: 231–234.

Semenza, G. L. and Wang, G. L. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol, *12:* 5447–5454.

Semenza, G. L., Roth, P. H., Fang, H. M., and Wang, G. L. 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem, *269:* 23757–23763.

Shaw, R. J. 2006. Glucose metabolism and cancer. Curr Opin Cell Biol, *18:* 598–608.

Shim, H., Dolde, C., Lewis, B. C., Wu, C. S., Dang, G., Jungmann, R. A., Dalla-Favera, R., and Dang, C. V. 1997. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci USA, *94:* 6658–6663.

Singh, D., Banerji, A. K., Dwarakanath, B. S., Tripathi, R. P., Gupta, J. P., Mathew, T. L., Ravindranath, T., and Jain, V. 2005. Optimizing cancer radiotherapy with 2-deoxy-d-glucose dose escalation studies in patients with glioblastoma multiforme. Strahlenther Onkol, *181:* 507–514.

Singh, K. K. 2004. Mitochondrial dysfunction is a common phenotype in aging and cancer. Ann NY Acad Sci, *1019:* 260–264.

Smith, T. A., Sharma, R. I., Thompson, A. M., and Paulin, F. E. 2006. Tumor 18F-FDG incorporation is enhanced by attenuation of P53 function in breast cancer cells in vitro. J Nucl Med, *47:* 1525–1530.

Sols, A. and Crane, R. K. 1954. Substrate specificity of brain hexokinase. J Biol Chem, *210:* 581–595.

Stiles, B., Groszer, M., Wang, S., Jiao, J., and Wu, H. 2004. PTENless means more. Dev Biol, *273:* 175–184.

Stryer, L. 1995. Biochemistry. New York: W. H. Freeman.

Taylor, R. W. and Turnbull, D. M. 2005. Mitochondrial DNA mutations in human disease. Nat Rev Genet, *6:* 389–402.

Thierbach, R., Schulz, T. J., Isken, F., Voigt, A., Mietzner, B., Drewes, G., von Kleist-Retzow, J. C., Wiesner, R. J., Magnuson, M. A., Puccio, H., Pfeiffer, A. F., Steinberg, P., and Ristow, M. 2005. Targeted disruption of hepatic frataxin expression causes impaired mitochondrial function, decreased life span, and tumor growth in mice. Hum Mol Genet, *14:* 3857–3864.

Timofeev, O., Lee, T. Y., and Bulavin, D. V. 2005. A subtle change in p38 MAPK activity is sufficient to suppress in vivo tumorigenesis. Cell Cycle, *4:* 118–120.

Vogelstein, B. and Kinzler, K. W. 1993. The multistep nature of cancer. Trends Genet, *9:* 138–141.

Warburg, O. 1930. The Metabolism of Tumours. London: Constable.

Warburg, O. 1931. The oxygen-transferring ferment of respiration. Nobel Lecture,

Warburg, O. 1956a. On the origin of cancer cells. Science, *123:* 309–314.

Warburg, O. 1956b. On respiratory impairment in cancer cells. Science, *124:* 269–270.

Warburg, O., Posener, K., and Negelein, E. 1924. Über den Stoffwechsel der Tumoren (On metabolism of tumors). Biochemische Zeitschrift, *152:* 319–344.

Warburg, O., Wind, F., and Negelein, E. 1926. The metabolism of tumors in the body. J Gen Physiol 519–530.

Weinhouse, S. 1956. On respiratory impairment in cancer cells. Science, *124:* 267–269.

Wu, M., Neilson, A., Swift, A. L., Moran, R., Tamagnine, J., Parslow, D., Armistead, S., Lemire, K., Orrell, J., Teich, J., Chomicz, S., and Ferrick, D. A. 2007. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. Am J Physiol Cell Physiol, *292:* C125-C136.

Xu, R. H., Pelicano, H., Zhou, Y., Carew, J. S., Feng, L., Bhalla, K. N., Keating, M. J., and Huang, P. 2005. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res, *65:* 613–621.

Younes, M., Lechago, L. V., and Lechago, J. 1996. Overexpression of the human erythrocyte glucose transporter occurs as a late event in human colorectal carcinogenesis and is associated with an increased incidence of lymph node metastases. Clin Cancer Res, *2:* 1151–1154.

Zhang, X. D., Deslandes, E., Villedieu, M., Poulain, L., Duval, M., Gauduchon, P., Schwartz, L., and Icard, P. 2006. Effect of 2-deoxy-D-glucose on various malignant cell lines in vitro. Anticancer Res, *26:* 3561–3566.

Zhou, S., Kachhap, S., and Singh, K. K. 2003. Mitochondrial impairment in p53-deficient human cancer cells. Mutagenesis, *18:* 287–292.

Zhu, Z., Jiang, W., McGinley, J. N., and Thompson, H. J. 2005. 2-Deoxyglucose as an energy restriction mimetic agent: effects on mammary carcinogenesis and on mammary tumor cell growth in vitro. Cancer Res, *65:* 7023–7030.

The Lipogenic Switch in Cancer

Koen Brusselmans and Johannes V. Swinnen

Introduction

Several decades ago it was observed that tumor tissues show a high rate of fatty acid synthesis. In contrast to normal cells, almost all fatty acids in cancer cells are derived from de novo synthesis, irrespective of the nutritional supply of lipids via the circulation. This enhanced lipogenic activity in tumors is associated with a markedly increased expression and/or activation of lipogenic enzymes. This phenomenon, referred to here as the lipogenic switch is found in nearly all examined cancer types, occurs very early in cancer development, and is more pronounced in more aggressive tumors, rendering changes in the lipogenic pathway one of the most common molecular changes associated with the development and progression of cancer. In this chapter we will review the evidence for the existence of a lipogenic switch in cancer, the mechanisms underlying this remarkable phenomenon, its role in cancer cell biology, and its potential for cancer intervention.

Lipids in Normal Cell Physiology

Lipids constitute an important class of biomolecules that are involved in numerous biological and biochemical processes. Lipids function as the major building blocks of biological membranes that separate the cellular content from the environment and compartmentalize many functions vital for the normal functioning of the cell. They serve as anchors to target proteins to membranes, thereby guiding enzymes and proteins to their appropriate environment for optimal functioning. Lipids serve as efficient energy stores in the human body, act as signaling molecules, or are involved in specific processes, including production of myelin in the nervous system, surfactant in the lungs, and milk lipids during lactation.

Under normal physiological conditions, most body cells obtain the majority of the required lipids via the circulation (Weiss et al. 1986). These lipid molecules are

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mostly derived from the diet and are further modified, stored, and released by the liver and by adipose tissue. Lipids are carried in the circulation in water-soluble lipoprotein complexes, which consist of a hydrophilic surface of free cholesterol, phospholipids, and apolipoproteins and a hydrophobic core containing triglycerides and cholesterol esters. Lipoprotein lipases (LPL) hydrolyze lipids of these lipoprotein complexes, resulting in the release of free fatty acids, which are bound to albumin in the plasma (Ginsberg 1998). Because of their lipophilic character, it was initially believed that fatty acids cross the cell membrane by passive diffusion. However, it has been demonstrated that the majority of the cellular fatty acid uptake occurs via protein-mediated transport (Doege and Stahl 2006; Bonen et al. 2007). Although the precise mechanism(s) by which fatty acids are transported into the cell are not fully understood, several membrane proteins are known to facilitate the uptake of fatty acids, including fatty acid transport proteins (FATP), fatty acid translocase (FAT/CD36), plasmalemmal fatty acid binding protein (FABPpm), and caveolin-1. Once inside the cell, fatty acids bind to FABP or they can also be directly converted by long-chain acyl-CoA synthetases (ACSL) into activated fatty acyl-CoA esters, which are then bound to acyl-CoA binding proteins (ACBP) (Doege and Stahl 2006; Bonen et al. 2007), thereby unloading the transporters. Fatty acids are used for the synthesis of phospholipids (the major building blocks of membranes), triglyceride synthesis and cholesterol esterification (storage), energy production (via beta-oxidation), or protein modification (via acylation) (Fig. 1). Cholesterol, another major component of membranes is taken up from low density lipoprotein (LDL) particles that bind to LDL receptors (Ginsberg 1998).

Besides lipid uptake, most human cells have the ability to synthesize a variety of lipids de novo. The main carbon source for the synthesis of lipids is glucose. First, in the glycolytic pathway, glucose is converted to pyruvate. Pyruvate enters the mitochondria, where it is metabolized to citrate in the Krebs cycle. Via the citrate–malate–antiport system citrate can reenter the cytoplasm, where it is converted by ATP citrate lyase (ACL) to acetyl-CoA, the main precursor of both fatty acids and cholesterol. The rate-limiting step in the fatty acid synthesis pathway is the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase-alpha (ACC-alpha). Acetyl-CoA and malonyl-CoA are then used by fatty acid synthase (FASN), an enzyme that catalyzes seven different reactions, to form saturated long-chain fatty acids such as palmitate (Fig. 1). These fatty acids can be further modified by elongases and desaturases. Importantly, human cells are not able to synthesize polyunstaurated fatty acids such as linoleic acid and alpha-linolenic acid, which have to be derived from the diet and are therefore referred to as essential fatty acids. In the cholesterol biosynthetic pathway, HMG-CoA synthase and HMG-CoA reductase convert acetyl-CoA to mevalonate. In a series of condensation reactions, mevalonate is then used to form farnesyl diphosphate, which is further modified by squalene synthase (SQS) and other enzymes resulting in the synthesis of cholesterol (Fig. 1) (Swinnen et al. 2006).

In humans, de novo lipogenesis is particularly active during embryonic development. Inhibition of fatty acid synthesis or cholesterol synthesis via targeted gene inactivation of FASN, ACC-alpha, HMG-CoA reductase, or SQS results in

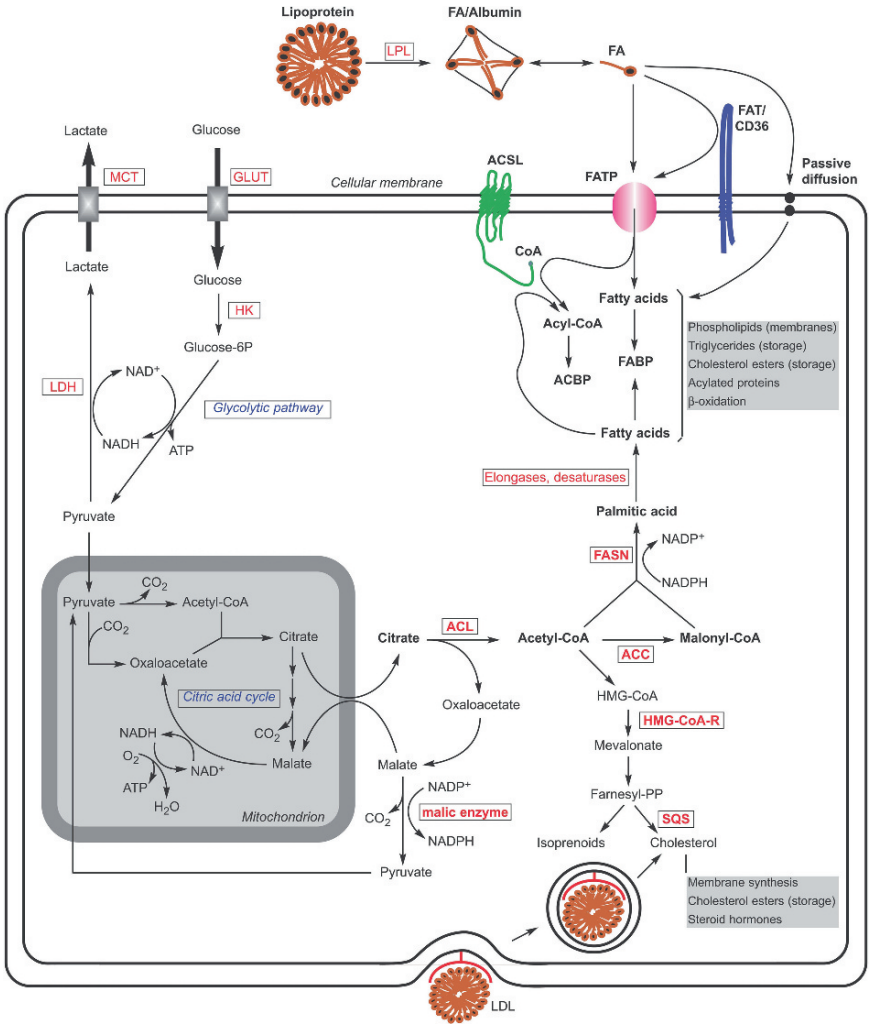

Fig. 1 Cellular uptake and de novo synthesis of lipids. Upon cellular uptake by glucose transporters (GLUT), glucose is phosphorylated by hexokinases (HK) and enters the glycolytic pathway generating pyruvate and ATP. In the mitochondria pyruvate is converted to acetyl-CoA, which enters the citric acid cycle, resulting in the formation of citrate. Citrate can be oxidized to carbon dioxide and oxaloacetate (generating ATP) or it can be transported to the cytosol, where it is converted to oxaloacetate and acetyl-CoA by ATP citrate lyase (ACL). Acetyl-CoA carboxylase-alpha (ACC) carboxylates acetyl-CoA to malonyl-CoA. In a cyclic condensation reaction requiring NADPH, FASN uses acetyl-CoA and malonyl-CoA to catalyze the synthesis of long-chain fatty acids (e.g. palmitate). Saturated long chain fatty acids can be further modified by elongases and/or desaturases. The reducing factor NADPH can be acquired via the hexose monophosphate shunt (not shown in the figure), or can be provided in a reaction catalyzed by malic enzyme, which converts cytosolic malate (originating from oxaloacetate generated by ACL) to carbon dioxide and pyruvate. In the mitochondria, pyruvate can be oxidized to carbon dioxide (citric acid cycle) or it can be carboxylated to replete the oxaloacetate pool. Under anaerobic conditions, pyruvate may

embryonic lethality (Tozawa et al. 1999; Chirala et al. 2003; Ohashi et al. 2003; Abu-Elheiga et al. 2005), illustrating the importance of these pathways in the developing embryo. Later in development and in the adult organism, the expression and activity of lipogenic enzymes is low in most body cells and is primarily related to specific functions and/or conditions (Weiss et al. 1986; Kuhajda 2000; Swinnen et al. 2006). Depending on the diet, liver and adipose tissue convert excess carbohydrate to fatty acids, which are stored as triglycerides in adipocytes. Active fatty acid synthesis also occurs in the lungs (for surfactant production), in the lactating breast (to produce fatty acids for milk lipids) and in the cycling endometrium (during the proliferative phase).

The Lipogenic Switch in Cancer Cells

Several decades ago it was observed that in contrast to normal tissues, tumor tissues show a high rate of fatty acid synthesis and that in cancer cells most lipids are derived from de novo synthesis, irrespective of the nutritional supply of lipids via the circulation (Medes et al. 1953; Sabine et al. 1967; Ookhtens et al. 1984). These changes were in part related to the marked activation of lipogenic enzymes, which is seen in several cancer tissues (Szutowicz et al. 1979; Turyn et al. 2003). During the last decade, an overwhelming body of evidence has been gathered for the overexpression of lipogenic enzymes in cancer cells. The enzyme that has been studied most exhaustively in this respect is FASN. In 1994, Kuhajda and coworkers found that the oncogenic antigen-519 (OA-519), a protein that was expressed at high levels in both breast and prostate cancer (Kuhajda et al. 1994), was identical to FASN. In the following years, using various independent antibodies against FASN,

Fig. 1 (Continued) function as electron acceptor, resulting in lactate dehydrogenase (LDH)-mediated production of lactate, which is excreted from the cell via monocarboxylate transporters (MCT). Besides de novo synthesis, another source of fatty acids is cellular uptake from lipoprotein particles in the circulation. After lipid hydrolysis by lipoprotein lipases (LPL), fatty acids (FA) are bound to albumin to increase their solubility. Fatty acids may cross the cell membrane by passive diffusion, but the majority of the cellular fatty acid uptake occurs via protein-mediated transport, which involves the action of fatty acid transport proteins (FATP) and fatty acid translocase (FAT/CD36). In the cytosol, fatty acids bind to fatty acid binding proteins (FABP) or they are directly converted by long-chain acyl-CoA synthetases (ACSL) into activated acyl-CoA esters, which are bound to acyl-CoA binding proteins (ACBP), thereby unloading the transporters. Fatty acids can be used in the cell for the synthesis of phospholipids (membranes), triglycerides or cholesterol esters (storage), for protein acylation (membrane anchorage) or for beta-oxidation (energy). Acetyl-CoA is also the main precursor for the synthesis of cholesterol and isoprenoids. First HMG-CoA is formed, which is converted to mevalonate by the action of HMG-CoA reductase (HMG-CoA-R). Mevalonate is further metabolized into farnesyl diphosphate, which can be used for the synthesis of isoprenoids or can be converted to cholesterol by squalene synthase (SQS) and other enzymes. Cholesterol can also be taken up by the cell via LDL receptor-mediated internalization of LDL particles. Cholesterol is an essential membrane compound and also functions as the precursor of steroid molecules (hormones); cholesterol can also be esterified for storage

overexpression of FASN was demonstrated in a wide variety of cancers, including cancer of the breast, prostate, ovary, endometrium, colon, thyroid, bladder, kidney, lung, liver, pancreas, stomach, oesophagus, oral tongue, oral cavity, and skin (Kuhajda 2000; Swinnen et al. 2006). More recently, increased levels of FASN were also observed in mesothelioma, retinoblastoma, glioma, and nephroblastoma, in Paget's disease of the vulva and in chemically and hormonally induced liver tumors of the rat (Swinnen et al. 2006; Zhao et al. 2006). In various cancer types, FASN expression is already enhanced in the earliest stages of cancer development (Piyathilake et al. 2000; Swinnen et al. 2002; Esslimani-Sahla et al. 2007). Furthermore, FASN expression gets more pronounced as the tumor progresses (Shurbaji et al. 1996; Kuhajda 2000; Kapur et al. 2005). In prostate cancer patients, elevated levels of FASN predict a poor outcome and significantly increase the risk of death (Shurbaji et al. 1996). Also in lung cancer, breast cancer, and melanoma, increased FASN expression has been associated with disease recurrence, metastasis, and poor survival (Alo et al. 1996; Visca et al. 2004; Kapur et al. 2005).

Various studies (including DNA microarray studies) have shown that tumor-associated overexpression of FASN not only occurs at the protein level but also at the mRNA level (Bull et al. 2001; Sebastiani et al. 2006; Prowatke et al. 2007). In addition, besides FASN, other lipogenic enzymes also, including ACC-alpha and ACL, show enhanced expression levels and activity in various types of cancer (Szutowicz et al. 1979; Milgraum et al. 1997; Moncur et al. 1998; Swinnen et al. 2000b; Turyn et al. 2003; Yahagi et al. 2005). Another interesting observation in this context is that in contrast to normal prostate epithelial cells, which typically show high intracellular citrate concentrations, citrate levels are markedly reduced in prostatic cancer cells (Costello and Franklin 1991a, b, 1997, 2005). These changes are related in part to differences in zinc accumulation and subsequent activation of aconitase, which increases citrate oxidation, but also may reflect an enhanced conversion of citrate into acetyl-CoA, which is used for lipogenesis. In support of this hypothesis, it was demonstrated that citrate enhanced the metastatic behavior of prostate cancer cells and that this induction depended on the activity of FASN (Mycielska et al. 2006).

Mechanism of the Lipogenic Switch in Cancer

From numerous studies, a very complex picture of the mechanism underlying the lipogenic switch has emerged. Initially, increased lipogenic gene expression in cancer cells was linked to aberrant growth factor signaling, which frequently occurs in many tumors. Growth factors that appeared to activate lipogenic pathways include epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and keratinocyte growth factor (KGF) (Swinnen et al. 2000a, 2006; Chang et al. 2005). Her2/neu, a receptor tyrosine kinase that is frequently overexpressed in breast cancer, may also be involved in upregulation of FASN in cancer. Expression of Her2/neu was observed to closely correlate with FASN

expression in breast cancer (Menendez et al. 2004a), and overexpression of Her2/neu-stimulated transcription of FASN and ACC-alpha in breast cancer cells (Kumar-Sinha et al. 2003; Yoon et al. 2007). This growth factor activation of lipogenesis in part is reminiscent of the activation of lipogenesis by insulin in liver and adipose tissue and has been shown to involve activation of sterol regulatory element-binding proteins (SREBPs) (Swinnen et al. 2000a; Chang et al. 2005; Swinnen et al. 2006). SREBPs are transcription factors that upon activation stimulate the expression of more than 30 lipogenic enzymes, thereby strictly controlling the cellular synthesis of fatty acids, cholesterol, triglycerides, and phospholipids (Horton et al. 2002). Signaling pathways that appear to be involved in this activation of SREBPs are the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3′-kinase (PI3K)/Akt pathway, which are often constitutively activated in tumor cells (Osaki et al. 2004). Yang et al. revealed that both inhibitors of MAPK and of PI3K downregulated SREBPs and decreased FASN levels in breast cancer cells (Yang et al. 2002). Furthermore, it was shown that mutation of PTEN, which occurs in many cancers and results in constitutive activation of Akt, stimulated the expression of FASN in cancer cells, in part mediated by SREBPs. Conversely, reintroduction of wild-type PTEN reduced FASN levels in cancer cells with a mutated PTEN gene (Van de Sande et al. 2002). Using an inducible form of Akt, Porstmann et al. also demonstrated that activation of Akt resulted in overexpression of FASN through activation of SREBPs (Porstmann et al. 2005). Importantly, it was shown that in clinical prostate cancers FASN overexpression was linked to activation of Akt, but inversely correlated with levels of PTEN (Bandyopadhyay et al. 2005; Van de Sande et al. 2005; Wang et al. 2005).

Akt may also activate lipogenesis in cancer cells by stimulating the translation of lipogenic enzymes such as FASN and ACC-alpha (Yoon et al. 2007) and by direct phosphorylation and activation of ACL, thereby directly promoting the supply of lipid precursors and, as a consequence, linking the glycolytic pathway to lipogenesis (Berwick et al. 2002; Bauer et al. 2005; Buzzai et al. 2005). Interestingly, in this respect, also glycolysis is enhanced by Akt in cancer. Akt increases the glycolytic rate (Elstrom et al. 2004; Altomare and Testa 2005) and stimulates the cellular uptake of glucose by promoting the activity and the recycling of glucose transporter-1 and by preventing its internalization (Wieman et al. 2007). In addition, Akt also modulates the intracellular localization of hexokinase-2, the predominant hexokinase isoform in cancer cells, which frequently shows tumor-associated overexpression. More particularly, Akt enhances binding of hexokinase-2 to the outer mitochondrial membrane, which allows to use the ATP generated in the mitochondria directly for glucose phosphorylation, thereby driving glucose instantly into the glycolytic pathway (Mathupala et al. 2006). Since Akt not only stimulates glycolysis and fatty acid synthesis but also protein synthesis (via the mTOR pathway) (Inoki et al. 2002; Hahn-Windgassen et al. 2005), the Akt-mediated increase in lipogenesis in cancer appears to be part of a more general coordinated metabolic switch, which enables cancer cells to survive and proliferate even under hypoxic and acidic conditions.

Besides Akt-mediated stimulation of lipogenesis, other mechanisms may also contribute to the increased expression and/or activity of lipogenic enzymes in cancer cells. Recently, Graner et al. showed that USP2a, an androgen-regulated gene that is frequently overexpressed in prostate cancer, increased the stability of FASN protein. The isopeptidase USP2a is an ubiquitin-specific protease that removes ubiquitin from particular proteins, thereby preventing their ubiquitin-mediated proteasomal degradation. Inactivation of USP2a significantly decreased FASN expression in cancer cells (Graner et al. 2004). Furthermore, Shah et al. observed that in ~25% of the examined prostate cancers the gene copy number of the FASN gene was increased (Shah et al. 2006). Another recent study has demonstrated that BRCA1 (breast cancer susceptibility gene 1), a scaffold protein involved in multiple cellular functions, binds to the phosphorylated (inactive) form of ACC-alpha (pACC-alpha), thereby preventing its dephosphorylation to the active form. Interestingly, BRCA1 is frequently mutated in a large number of hereditary breast cancers. Several germline BRCA1 mutations in breast cancer have been shown to result in a loss of interaction between BRCA1 and pACC-alpha. These mutations may enhance lipogenesis as the inactive pACC-alpha is no longer protected from dephosphorylation and can be converted into the active ACC-alpha (Magnard et al. 2002). It was demonstrated that overexpression of BRCA1 decreased de novo synthesis of fatty acids, whereas RNAi-mediated silencing of BRCA1 increased fatty acid synthesis in breast cancer cells (Moreau et al. 2006). Consequently, mutation of BRCA1 and subsequent dephosphorylation and activation of ACC-alpha may also contribute to increased lipogenesis in breast cancer. Another factor that may be in involved in increased lipogenesis in cancer cells is Spot 14 (S14), a carbohydrate- and thyroid hormone-inducible nuclear protein that activates genes encoding the enzymes of the fatty acid synthesis in liver and adipose tissue. Spot 14 was shown to be strongly expressed in breast cancer. Knockdown of Spot 14 significantly reduced lipid synthesis in breast cancer cells, indicating a possible role of Spot 14 in breast cancer-related overexpression of lipogenic genes (Moncur et al. 1998; Kinlaw et al. 2006).

In steroid-responsive tumors such as prostate and breast tumors, lipogenesis is markedly enhanced by steroid hormones. In prostate cancer cells, androgens stimulate expression of FASN via activation of SREBPs. It has been demonstrated that androgens increase the expression of SCAP, a protein that escorts SREBP precursors from the endoplasmic reticulum to the Golgi bodies. In the Golgi body, SREBP precursors are cleaved into their active forms, which migrate to the nucleus and bind to sterol response elements in promoters of target genes (Horton et al. 2002; Swinnen et al. 2004; Heemers et al. 2006). Recently, the SCAP gene has been shown to contain an androgen responsive element, which allows androgen-induced expression of SCAP (Heemers et al. 2004). In addition, androgens also cause a shift in the isoform expression of Insig-1 and Insig-2, retention proteins that interact with SCAP and retain the SCAP–SREBP complexes in the endoplasmic reticulum (Heemers et al. 2006). Most likely, the androgen-mediated upregulation of SCAP and shift in Insig isoform expression disturb the normal balance between SCAP and Insig, thereby favoring translocation of the SCAP–SREBP complex to the Golgi

body, where SREBP is proteolytically activated. Also, expression of other lipogenic enzymes, including ACC-alpha, ACL, and several enzymes involved in cholesterol synthesis, is induced by androgens in prostate cancer cells (Swinnen et al. 1997). In addition to activation of SREBPs, androgens also stimulate the expression of USP2a (Graner et al. 2004), thereby further stabilizing the FASN protein. Furthermore, besides androgens, also progestagens have been described to stimulate expression of lipogenic genes in cancer cells (Chalbos et al. 1990). Interestingly, both androgen- and progestagen-mediated induction of lipogenesis were shown to be accompanied by a marked increase in the expression of Spot 14 in breast cancer cells, suggesting that steroid hormones may also enhance lipid synthesis in cancer cells via induction of the lipogenic gene activator Spot 14 (Heemers et al. 2000). Taken together, these studies provide a complex picture of the mechanism(s) underlying the lipogenic switch, affecting lipogenic enzymes at all levels of regulation.

Lipogenic Enzymes as Potential Targets for Antineoplastic Intervention

The marked overexpression and/or activation of lipogenic enzymes in a wide variety of human tumors and its correlation with tumor prognosis has raised the questions "which specific tumor-related functions are associated with increased lipogenesis" and "can cancer-related increased lipogenic gene expression be exploited in the search for new anticancer therapies ?"

Interestingly, several studies using chemical inhibitors or RNA interference have shown that inhibition of the fatty acid synthesis pathway attenuates proliferation and induces cell death in various cancer cell lines in vitro as well as in tumor xenografts in vivo. Initially the mycotoxin cerulenin, isolated from *Cephalosporium caerulens*, which restricts the growth of bacteria by inhibiting fatty acid synthesis (Omura 1976), was demonstrated not only to inhibit FASN in several human breast and colon cancer cell lines but also to induce growth arrest and apoptosis of these cells. In contrast, cerulenin had only little effect on non-tumoral fibroblasts, displaying low levels of endogenous fatty acid synthesis (Kuhajda et al. 1994; Pizer et al. 1996a, c, 1998; Li et al. 2000; Thupari et al. 2001; Menendez et al. 2004b). Moreover, cerulenin also markedly reduced fatty acid biosynthesis in ovarian xenografts in mice, delayed disease progression, and increased animal survival (Pizer et al. 1996b). On the basis of these findings, FASN was proposed as a new potential target for antineoplastic therapy.

Because of its chemical instability and the presence of a highly reactive epoxide group (which may induce nonspecific binding), the possible use of cerulenin for therapeutic treatments is, however, limited. Therefore, Kuhajda and colleagues developed synthetic analogues of cerulenin, the compounds C75 and C93. Similar to cerulenin, C75 and C93 blocked fatty acid synthesis, inhibited growth, and

induced cell death in various cancer cells in cultures in vitro and in xenograft in vivo (Pizer et al. 1998, 2000; Kuhajda et al. 2000; Gabrielson et al. 2001; Li et al. 2001; Zhou et al. 2003, 2007; Menendez et al. 2004b). C75 also delayed tumor progression in the neu-N transgenic mouse model of mammary cancer (Alli et al. 2005). Besides C75 and C93, several other chemical inhibitors of FASN have been identified. Tea polyphenolic compounds and several other flavonoids, which have been shown to act as inhibitors of FASN, also inhibited lipogenesis and induced growth arrest and death in cancer cells (Wang and Tian 2001; Brusselmans et al. 2003, 2005b; Yeh et al. 2003; Li and Tian 2004). Interestingly, viability of non-tumoral fibroblasts was not affected by these polyphenols. In addition, the anti-bacterial compound triclosan, which inhibits the enoyl reductase domain of FASN, was cytotoxic to breast cancer cells and suppressed methylnitrosurea-induced carcinogenesis in Sprague-Dawley rats (Liu et al. 2002; Lu and Archer 2005). Furthermore, in an activity-based screening for inhibitors of serine hydrolases, the anti-obesity drug orlistat was identified as an inhibitor of the thioesterase domain of FASN. Orlistat not only inhibited fatty acid synthesis and induced growth arrest and death in prostate cancer cells, but also delayed tumor progression of xenografts in mice (Kridel et al. 2004; Menendez et al. 2005b). Interestingly, FASN inhibition (using chemical inhibitors or RNAi) also sensitized tumor cells for chemotherapeutic compounds such as paclitaxel (Menendez et al. 2005a) and for signal transduction inhibitors such as the PI3K inhibitor LY294002 (Wang et al. 2005).

It needs to be mentioned that most of these chemical inhibitors do not show complete specificity in their FASN inhibitory activity: cerulenin also inhibits cholesterol synthesis (Malvoisin and Wild 1990) and protein palmitoylation (Lawrence et al. 1999; De Vos et al. 2001); C75 induces weight loss and visceral illness (Loftus et al. 2000; Clegg et al. 2002), acts as a nonspecific neuronal activator (Takahashi et al. 2004), and stimulates carnitine palmitoyltransferase-1 activity (Thupari et al. 2002); flavonoid polyphenols inhibit several kinases and topoisomerases and also act as antioxidants (Middleton et al. 2000; Yang et al. 2001). To circumvent possible side-effects of chemical inhibitors, several groups have made use of a recently developed genetic tool, RNA interference, to specifically silence lipogenic enzymes. RNAi-mediated inhibition of FASN was shown to block fatty acid synthesis and to induce growth arrest and apoptosis in prostate and breast cancer cells (De Schrijver et al. 2003; Menendez et al. 2005a, c). In contrast, non-tumoral fibroblasts showed normal growth and viability after inhibition of FASN (De Schrijver et al. 2003).

With respect to the mechanism by which cancer cells stop proliferating and ultimately die upon inhibition of FASN, Knowles and Smith performed a micro-array analysis on breast cancer cells and showed that RNAi-mediated inhibition of FASN resulted in a downregulation of genes involved in lipogenesis, glycolysis, Krebs cycle, and oxidative phosphorylation, and in an upregulation of pathways involved in cell cycle arrest and apoptosis (Knowles and Smith 2007). Other studies also showed that cancer cell death induced by FASN inhibition was primarily mediated by apoptosis (Kuhajda 2006; Swinnen et al. 2006). The observation that the caspase inhibitor z-VAD-fmk suppressed cancer cell death after RNAi-mediated

FASN silencing, suggests a role for caspases in the cytotoxic effects mediated by FASN inhibition (Brusselmans et al. 2005a). However, the precise mechanisms that lead to the activation of the apoptotic pathway remain largely elusive. Recently, Kuhajda and coworkers documented that in ovarian cancer AMPK activity was essential for the cytotoxic effect induced by FASN inhibition. Using the chemical FASN inhibitor C93, they showed that FASN inhibition activated AMPK activity and caused apoptosis of ovarian cancer cells in vitro and in vivo. Pretreatment with the AMPK inhibitor compound C rescued cancer cells from C93 cytotoxicity, indicating that the cytotoxic effects were mediated by AMPK (Zhou et al. 2007). The ACC-alpha inhibitor TOFA did not induce cancer cell death, suggesting that accumulation of FASN substrate was the trigger for AMPK activation by FASN inhibition. Kuhajda and colleagues had previously found that blockage of FASN in cancer cells resulted in an accumulation of the fatty acid precursor malonyl-CoA (Pizer et al. 2000; Thupari et al. 2001). The increased intracellular levels of malonyl-CoA and subsequent inactivation of fatty acid oxidation were thought to be the cause of the observed cytotoxicity induced by FASN inhibition, since simultaneous treatment of cancer cells with the FASN inhibitor C75 and the ACC-alpha inhibitor TOFA did not induce any cytotoxic effects (Pizer et al. 2000). More recently, Bandyopadhyay et al. (2006) showed that RNAi-mediated inhibition of FASN increased intracellular levels of ceramides and induced expression of the proapoptotic genes BNIP3, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), and death-associated protein kinase-2 in breast cancer cells. Furthermore, blockage of fatty acid beta-oxidation via inhibition of carnitine palmitoyltransferase-1 (CPT-1), the enzyme that mediates transport of fatty acids into the mitochondria for oxidation, also resulted in increased ceramide levels and BNIP3 induction in breast cancer cells. Interestingly, treatment with a ceramide synthase inhibitor or with small interfering RNA targeting BNIP3 rescued cancer cells from apoptosis induced by FASN inhibition. As the ACC-alpha inhibitor TOFA abrogated the increase in ceramide induced by FASN knockdown, the authors postulated that accumulation of malonyl-CoA was involved: FASN inhibition results in elevated levels of malonyl-CoA, which inhibit CPT-1 and increase ceramide levels, thereby initiating the apoptotic machinery in cancer cells (Bandyopadhyay et al. 2006). As cancer cells frequently show a coordinated upregulation of lipogenic enzymes, the overall high activity of this pathway and therefore high accumulation of malonyl-CoA upon FASN inhibition is thought to play a key role in the cancer cell-selective effects of FASN inhibitors.

Recently, it has been shown that, besides inhibition of FASN, RNAi-mediated inhibition of ACC-alpha also markedly decreases lipogenesis, attenuates proliferation, and induces death both in prostate and breast cancer cells (Brusselmans et al. 2005a; Chajes et al. 2006). Furthermore, administration of exogenous palmitate, the most important end product of FASN, suppresses the cytotoxic effects of RNAi-mediated inhibition of FASN as well as of ACC-alpha in prostate cancer cells (Brusselmans et al. 2005a), strongly indicating that (at least in vitro) depletion of fatty acids per se may be sufficient to cause cell death upon inhibition of fatty acid synthesis in cancer cells.

Besides inhibition of FASN and ACC-alpha, also chemical and RNAi-mediated inhibition of ACL, the enzyme that provides cytosolic acetyl-CoA, which is the main precursor for lipid synthesis, decreased lipogenesis and limited in vitro growth and survival of lung cancer cells (Hatzivassiliou et al. 2005). The chemical ACL inhibitor SB-204990 also impaired growth of tumor xenografts in vivo, with tumor cells showing a high glycolytic rate being significantly more sensitive to ACL inhibition (Hatzivassiliou et al. 2005). Since cytosolic acetyl-CoA not only functions as a building block for fatty acids but is also the main precursor for the mevalonate pathway (Sato and Takano 1995), blockage of ACL may also affect cholesterol biosynthesis and protein isoprenylation. Therefore, it cannot be excluded that part of the effects mediated by ACL inhibition in cancer cells was the result of blockage of the mevalonate pathway. Although there is no evidence for a general increase of cholesterol biosynthetic gene expression and/or activity in cancer, it has been shown that statins, cholesterol-lowering drugs, which inhibit HMG-CoA reductase activity (Veillard and Mach 2002), induce growth arrest and apoptosis in various tumor cell lines in vitro (Denoyelle et al. 2003; Zhong et al. 2003; Muck et al. 2004; Khanzada et al. 2006). Furthermore, population-based studies indicate that statins may also reduce the risk to develop several cancers (Demierre et al. 2005). Since statins inhibit the synthesis of mevalonate, they not only block cholesterol synthesis but also protein isoprenylation (Veillard and Mach 2002), which is required for the cancer-associated activity of signaling proteins such as Ras (Sebti 2005). Specific inhibitors of farnesylation and geranylgeranylation were shown to inhibit cancer cell growth and survival (Xia et al. 2001), indicating that protein isoprenylation indeed plays an essential role in tumorigenesis. However, RNAi-mediated and chemical inhibition of SQS, the first enzyme of the mevalonate/isoprenoid pathway that is exclusively committed to the synthesis of sterols, was recently also shown to limit proliferation and induce apoptosis in prostate cancer cells (Brusselmans et al. 2007). These findings indicate that besides fatty acid synthesis cholesterol synthesis also plays an essential role in cancer cell growth and survival and may also be considered as a potential new target for anticancer therapy.

Interestingly, knockdown of Spot 14, a regulator of lipogenic gene expression, also significantly reduced lipid synthesis and caused apoptosis in breast cancer cells (Moncur et al. 1998; Kinlaw et al. 2006). Accordingly, Spot 14 has been proposed as a potential therapeutic target to prevent and/or treat cancer.

With regard to the selective cytotoxic effects of inhibition of lipogenic enzymes in cancer cells but not in normal cells (De Schrijver et al. 2003; Brusselmans et al. 2005a; Brusselmans et al. 2005b; Chajes et al. 2006), it has been hypothesized that changes in the expression of lipoprotein lipase (LPL), the enzyme that releases fatty acids from lipoprotein complexes, may play a key role. In fact, up to 68% of prostate tumors show a deletion of the gene locus encoding LPL (Bova et al. 1993). Furthermore, in breast cancer, the relative depletion of adipocytes, which represent the main source of LPL in normal breast tissue, may lead to an increased dependence on endogenous lipogenesis (Kinlaw et al. 2006). Further research is certainly needed to investigate this hypothesis.

Impact of Increased Lipogenesis on Tumor Cell Biology

Although numerous studies suggest that fatty acid synthesis is an important process in tumorigenesis, the precise role of the increased lipogenesis in cancer is not fully understood. Incorporation assays with ^{14}C-acetate have shown that the majority of the newly synthesized fatty acids in cancer cells are incorporated into phospholipids, with phosphatidylcholine being the most abundant lipid. Inhibition of FASN or ACC-alpha significantly reduced lipid synthesis in cancer cells and affected in the first place the cellular levels of phospholipids and in particular of phosphatidylcholine (De Schrijver et al. 2003; Swinnen et al. 2003; Brusselmans et al. 2005a; Brusselmans et al. 2005b). Interestingly, choline kinase, an enzyme involved in the synthesis of phosphatidylcholine, is also frequently overexpressed in a variety of human cancers (Ramirez de Molina et al. 2002). Moreover, inhibition of choline kinase or blockage of other steps in the phospholipid synthesis pathway also induced apoptosis in cancer cells (Cui et al. 1996; van der Sanden et al. 2003; Rodriguez-Gonzalez et al. 2005), similarly as observed after inhibition of fatty acid biosynthetic enzymes. These findings indicate that cancer cells depend on de novo synthesis of phospholipids, the major building blocks of cellular membranes, for their growth and survival. This dependence may reflect the increased demand of rapidly proliferating cells for lipids and the decreased access to circulating lipids for instance caused by changes in LPL expression. Besides quantitative aspects of the lipogenic switch, activation of de novo lipogenesis in cancer cells, may also affect the lipid composition of membranes. In fact, in contrast to the diet-derived lipids, which also contain polyunsaturated fatty acids, newly synthesized fatty acids in cancer cells are mostly saturated or mono-unsaturated (Swinnen et al. 2003). As a consequence, the increased lipogenic activity in cancer cells may induce significant qualitative changes in the composition of the cellular phospholipids and as a result also of the cellular membranes. Indeed, it has been demonstrated that membranes of colon cancer cells show an increased ratio of saturated over unsaturated C18 fatty acids (Rakheja et al. 2005). In this context, it is worth mentioning that loss of stearoyl-CoA desaturase expression, an enzyme that converts saturated fatty acids to mono-unsaturated fatty acids, is a frequent event in prostate cancer, which may also contribute to increased levels of saturated fatty acids (Moore et al. 2005). Importantly, detergent-resistant membrane microdomains (also referred to as lipid rafts) mostly consist of cholesterol and of phospholipids with a high saturation degree (Simons and Ikonen 1997). Therefore, increased lipogenic activity in cancer cells may have a significant impact on the formation, composition, and function of these lipid rafts, which have been implicated in signal transduction, intracellular trafficking, cell polarization, and cell migration – key cellular processes relevant to cancer (Simons and Toomre 2000; Hancock 2006). It was observed that RNAi-mediated FASN inhibition in cancer cells (which ultimately resulted in apoptosis induction) preferentially reduced the cellular amount of raft-associated phospholipids, whereas non-raft lipids were much less affected (Swinnen et al. 2003). These findings further suggest an important role for cancer-associated lipogenesis in lipid

raft-mediated processes. In this context, it is tempting to propose that the inability of Orlistat-treated HUVEC cells to display VEGF-receptor-2 (VEGFR-2), a membrane-associated receptor tyrosine kinase, at the cell surface (Browne et al. 2006) is possibly the result of altered and/or decreased raft formation/composition (due to FASN inhibition), which may be essential for optimal VEGFR-2 localization and functioning.

Importantly, several studies showed that inhibition of cholesterol synthesis or removal of cholesterol from cellular membranes, thereby reducing lipid raft cholesterol levels, also inhibited raft function and resulted in growth retardation and death induction in cancer cells (Zhuang et al. 2002; Bang et al. 2005; Zhuang et al. 2005; Hager et al. 2006). Although there is no evidence for a general upregulation of cholesterol biosynthetic gene expression and/or activity in human cancer as it has been documented for FASN, these studies highlight the significance of the cholesterol synthesis pathway and confirm the importance of membrane composition and lipid raft functioning for tumor cell biology.

It should be taken into account that the increased lipogenic activity in cancer may not only affect plasma membranes but also membranes of intracellular organelles (e.g. mitochondria, nucleus, endoplasmic reticulum, Golgi vesicles). It was shown that FASN inhibition resulted in the induction of endoplasmic reticulum stress in tumor cells (Little et al. 2007). Furthermore, it has been proposed that the increased synthesis of fatty acids and lipids possibly plays a role in the maintenance of the redox balance in cancer cells with a high glycolytic rate under hypoxic conditions (Hochachka et al. 2002). Finally, increased lipogenesis in cancer cells may also affect protein acylation, a process that promotes proper localization (to membranes or rafts) and functioning of many distinct membrane proteins. However, although many functions have been proposed for the increased lipogenic activity in cancer cells, further investigation is needed to fully elucidate the precise role and implications of this phenomenon.

Conclusion

The described findings illustrate that enhanced expression and activity of lipogenic enzymes is a widespread phenomenon in many human cancers. This increased lipogenesis is mediated by modulation of several different lipogenic enzymes at various levels (increased copy number, enhanced transcription, protein stabilization, enhanced enzymatic activity) and contributes to the formation and functioning of membranes. Importantly, interfering with the lipogenic switch selectively attenuates tumor cell growth and survival, rendering lipogenic enzymes interesting targets for antineoplastic therapy.

Abbreviations

ACBP	Acyl-CoA binding protein
ACC-alpha	Acetyl-CoA carboxylase-alpha
ACL	ATP citrate lyase
AMPK	Adenosine monophosphate-activated kinase
BRCA1	Breast cancer susceptibility gene 1
CPT-1	Carnitine palmitoyltransferase-1
FABP	Fatty acid binding protein
FASN	Fatty acid synthase
FATP	Fatty acid transport protein
HMG-CoA	Hydroxymethylglutaryl-CoA
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
PI3K	Phosphaditylinositol 3′-kinase
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
RNAi	RNA interference
SCAP	SREBP cleavage-activating protein
SREBP	Sterol regulatory element-binding protein
TOFA	5-(Tetradecyloxy)-2-furoic acid

References

Abu-Elheiga, L., Matzuk, M. M., Kordari, P., Oh, W., Shaikenov, T., Gu, Z. and Wakil, S. J. 2005. Mutant mice lacking acetyl-CoA carboxylase 1 are embryonically lethal. Proc Natl Acad Sci USA 102: 12011–12016.

Alli, P. M., Pinn, M. L., Jaffee, E. M., McFadden, J. M. and Kuhajda, F. P. 2005. Fatty acid synthase inhibitors are chemopreventive for mammary cancer in neu-N transgenic mice. Oncogene 24: 39–46.

Alo, P. L., Visca, P., Marci, A., Mangoni, A., Botti, C. and Di Tondo, U. 1996. Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients. Cancer 77: 474–482.

Altomare, D. A. and Testa, J. R. 2005. Perturbations of the AKT signaling pathway in human cancer. Oncogene 24: 7455–7464.

Bandyopadhyay, S., Pai, S. K., Watabe, M., Gross, S. C., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Saito, K., Markwell, S. J., Wang, Y., Huggenvik, J., Pauza, M. E., Iiizumi, M. and Watabe, K. 2005. FAS expression inversely correlates with PTEN level in prostate cancer and a PI 3-kinase inhibitor synergizes with FAS siRNA to induce apoptosis. Oncogene 24: 5389–5395.

Bandyopadhyay, S., Zhan, R., Wang, Y., Pai, S. K., Hirota, S., Hosobe, S., Takano, Y., Saito, K., Furuta, E., Iiizumi, M., Mohinta, S., Watabe, M., Chalfant, C. and Watabe, K. 2006. Mechanism of apoptosis induced by the inhibition of fatty acid synthase in breast cancer cells. Cancer Res 66: 5934–5940.

Bang, B., Gniadecki, R. and Gajkowska, B. 2005. Disruption of lipid rafts causes apoptotic cell death in HaCaT keratinocytes. Exp Dermatol 14: 266–272.

Bauer, D. E., Hatzivassiliou, G., Zhao, F., Andreadis, C. and Thompson, C. B. 2005. ATP citrate lyase is an important component of cell growth and transformation. Oncogene 24: 6314–6322.

Berwick, D. C., Hers, I., Heesom, K. J., Moule, S. K. and Tavare, J. M. 2002. The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. J Biol Chem 277: 33895–33900.

Bonen, A., Chabowski, A., Luiken, J. J. and Glatz, J. F. 2007. Is membrane transport of FFA mediated by lipid, protein, or both? Mechanisms and regulation of protein-mediated cellular fatty acid uptake: molecular, biochemical, and physiological evidence. Physiology (Bethesda) 22: 15–29.

Bova, G. S., Carter, B. S., Bussemakers, M. J., Emi, M., Fujiwara, Y., Kyprianou, N., Jacobs, S. C., Robinson, J. C., Epstein, J. I., Walsh, P. C. et al. 1993. Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer. Cancer Res 53: 3869–3873.

Browne, C. D., Hindmarsh, E. J. and Smith, J. W. 2006. Inhibition of endothelial cell proliferation and angiogenesis by orlistat, a fatty acid synthase inhibitor. Faseb J 20: 2027–2035.

Brusselmans, K., De Schrijver, E., Heyns, W., Verhoeven, G. and Swinnen, J. V. 2003. Epigallocatechin-3-gallate is a potent natural inhibitor of fatty acid synthase in intact cells and selectively induces apoptosis in prostate cancer cells. Int J Cancer 106: 856–862.

Brusselmans, K., De Schrijver, E., Verhoeven, G. and Swinnen, J. V. 2005a. RNA interference-mediated silencing of the acetyl-CoA-carboxylase-alpha gene induces growth inhibition and apoptosis of prostate cancer cells. Cancer Res 65: 6719–6725.

Brusselmans, K., Vrolix, R., Verhoeven, G. and Swinnen, J. V. 2005b. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem 280: 5636–5645.

Brusselmans, K., Timmermans, L., Van de Sande, T., Van Veldhoven, P. P., Guan, G., Shechter, I., Claessens, F., Verhoeven, G. and Swinnen, J. V. 2007. Squalene synthase, a determinant of Raft-associated cholesterol and modulator of cancer cell proliferation. J Biol Chem 282: 18777–18785.

Bull, J. H., Ellison, G., Patel, A., Muir, G., Walker, M., Underwood, M., Khan, F. and Paskins, L. 2001. Identification of potential diagnostic markers of prostate cancer and prostatic intraepithelial neoplasia using cDNA microarray. Br J Cancer 84: 1512–1519.

Buzzai, M., Bauer, D. E., Jones, R. G., Deberardinis, R. J., Hatzivassiliou, G., Elstrom, R. L. and Thompson, C. B. 2005. The glucose dependence of Akt-transformed cells can be reversed by pharmacologic activation of fatty acid beta-oxidation. Oncogene 24: 4165–4173.

Chajes, V., Cambot, M., Moreau, K., Lenoir, G. M. and Joulin, V. 2006. Acetyl-CoA carboxylase alpha is essential to breast cancer cell survival. Cancer Res 66: 5287–5294.

Chalbos, D., Joyeux, C., Escot, C., Galtier, F. and Rochefort, H. 1990. Progestin-induced fatty acid synthetase in breast cancer. From molecular biology to clinical applications. Ann N Y Acad Sci 595: 67–73.

Chang, Y., Wang, J., Lu, X., Thewke, D. P. and Mason, R. J. 2005. KGF induces lipogenic genes through a PI3K and JNK/SREBP-1 pathway in H292 cells. J Lipid Res 46: 2624–2635.

Chirala, S. S., Chang, H., Matzuk, M., Abu-Elheiga, L., Mao, J., Mahon, K., Finegold, M. and Wakil, S. J. 2003. Fatty acid synthesis is essential in embryonic development: Fatty acid synthase null mutants and most of the heterozygotes die in utero. Proc Natl Acad Sci USA 100: 6358–6363.

Clegg, D. J., Wortman, M. D., Benoit, S. C., McOsker, C. C. and Seeley, R. J. 2002. Comparison of central and peripheral administration of C75 on food intake, body weight, and conditioned taste aversion. Diabetes 51: 3196–3201.

Costello, L. C. and Franklin, R. B. 1991a. Concepts of citrate production and secretion by prostate: 2. Hormonal relationships in normal and neoplastic prostate. Prostate 19: 181–205.

Costello, L. C. and Franklin, R. B. 1991b. Concepts of citrate production and secretion by prostate. 1. Metabolic relationships. Prostate 18: 25–46.

Costello, L. C. and Franklin, R. B. 1997. Citrate metabolism of normal and malignant prostate epithelial cells. Urology 50: 3–12.

Costello, L. C. and Franklin, R. B. 2005. 'Why do tumour cells glycolyse?': From glycolysis through citrate to lipogenesis. Mol Cell Biochem 280: 1–8.

Cui, Z., Houweling, M., Chen, M. H., Record, M., Chap, H., Vance, D. E. and Terce, F. 1996. A genetic defect in phosphatidylcholine biosynthesis triggers apoptosis in Chinese hamster ovary cells. J Biol Chem 271: 14668–14671.

De Schrijver, E., Brusselmans, K., Heyns, W., Verhoeven, G. and Swinnen, J. V. 2003. RNA interference-mediated silencing of the fatty acid synthase gene attenuates growth and induces morphological changes and apoptosis of LNCaP prostate cancer cells. Cancer Res 63: 3799–3804.

De Vos, M. L., Lawrence, D. S. and Smith, C. D. 2001. Cellular pharmacology of cerulenin analogs that inhibit protein palmitoylation. Biochem Pharmacol 62: 985–995.

Demierre, M. F., Higgins, P. D., Gruber, S. B., Hawk, E. and Lippman, S. M. 2005. Statins and cancer prevention. Nat Rev Cancer 5: 930–942.

Denoyelle, C., Albanese, P., Uzan, G., Hong, L., Vannier, J. P., Soria, J. and Soria, C. 2003. Molecular mechanism of the anti-cancer activity of cerivastatin, an inhibitor of HMG-CoA reductase, on aggressive human breast cancer cells. Cell Signal 15: 327–338.

Doege, H. and Stahl, A. 2006. Protein-mediated fatty acid uptake: Novel insights from in vivo models. Physiology (Bethesda) 21: 259–268.

Elstrom, R. L., Bauer, D. E., Buzzai, M., Karnauskas, R., Harris, M. H., Plas, D. R., Zhuang, H., Cinalli, R. M., Alavi, A., Rudin, C. M. and Thompson, C. B. 2004. Akt stimulates aerobic glycolysis in cancer cells. Cancer Res 64: 3892–3899.

Esslimani-Sahla, M., Thezenas, S., Simony-Lafontaine, J., Kramar, A., Lavaill, R., Chalbos, D. and Rochefort, H. 2007. Increased expression of fatty acid synthase and progesterone receptor in early steps of human mammary carcinogenesis. Int J Cancer 120: 224–229.

Gabrielson, E. W., Pinn, M. L., Testa, J. R. and Kuhajda, F. P. 2001. Increased fatty acid synthase is a therapeutic target in mesothelioma. Clin Cancer Res 7: 153–157.

Ginsberg, H. N. 1998. Lipoprotein physiology. Endocrinol Metab Clin North Am 27: 503–519.

Graner, E., Tang, D., Rossi, S., Baron, A., Migita, T., Weinstein, L. J., Lechpammer, M., Huesken, D., Zimmermann, J., Signoretti, S. and Loda, M. 2004. The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. Cancer Cell 5: 253–261.

Hager, M. H., Solomon, K. R. and Freeman, M. R. 2006. The role of cholesterol in prostate cancer. Curr Opin Clin Nutr Metab Care 9: 379–385.

Hahn-Windgassen, A., Nogueira, V., Chen, C. C., Skeen, J. E., Sonenberg, N. and Hay, N. 2005. Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. J Biol Chem 280: 32081–32089.

Hancock, J. F. 2006. Lipid rafts: Contentious only from simplistic standpoints. Nat Rev Mol Cell Biol 7: 456–462.

Hatzivassiliou, G., Zhao, F., Bauer, D. E., Andreadis, C., Shaw, A. N., Dhanak, D., Hingorani, S. R., Tuveson, D. A. and Thompson, C. B. 2005. ATP citrate lyase inhibition can suppress tumor cell growth. Cancer Cell 8: 311–321.

Heemers, H., Vanderhoydonc, F., Heyns, W., Verhoeven, G. and Swinnen, J. V. 2000. Progestins and androgens increase expression of Spot 14 in T47-D breast tumor cells. Biochem Biophys Res Commun 269: 209–212.

Heemers, H., Verrijdt, G., Organe, S., Claessens, F., Heyns, W., Verhoeven, G. and Swinnen, J. V. 2004. Identification of an androgen response element in intron 8 of the sterol regulatory element-binding protein cleavage-activating protein gene allowing direct regulation by the androgen receptor. J Biol Chem 279: 30880–30887.

Heemers, H. V., Verhoeven, G. and Swinnen, J. V. 2006. Androgen activation of the sterol regulatory element-binding protein pathway: Current insights. Mol Endocrinol 20: 2265–2277.

Hochachka, P. W., Rupert, J. L., Goldenberg, L., Gleave, M. and Kozlowski, P. 2002. Going malignant: The hypoxia-cancer connection in the prostate. Bioessays 24: 749–757.

Horton, J. D., Goldstein, J. L. and Brown, M. S. 2002. SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109: 1125–1131.

Inoki, K., Li, Y., Zhu, T., Wu, J. and Guan, K. L. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 4: 648–657.
Kapur, P., Rakheja, D., Roy, L. C. and Hoang, M. P. 2005. Fatty acid synthase expression in cutaneous melanocytic neoplasms. Mod Pathol 18: 1107–1112.
Khanzada, U. K., Pardo, O. E., Meier, C., Downward, J., Seckl, M. J. and Arcaro, A. 2006. Potent inhibition of small-cell lung cancer cell growth by simvastatin reveals selective functions of Ras isoforms in growth factor signalling. Oncogene 25: 877–887.
Kinlaw, W. B., Quinn, J. L., Wells, W. A., Roser-Jones, C. and Moncur, J. T. 2006. Spot 14: A marker of aggressive breast cancer and a potential therapeutic target. Endocrinology 147: 4048–4055.
Knowles, L. M. and Smith, J. W. 2007. Genome-wide changes accompanying knockdown of fatty acid synthase in breast cancer. BMC Genomics 8: 168.
Kridel, S. J., Axelrod, F., Rozenkrantz, N. and Smith, J. W. 2004. Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. Cancer Res 64: 2070–2075.
Kuhajda, F. P., Jenner, K., Wood, F. D., Hennigar, R. A., Jacobs, L. B., Dick, J. D. and Pasternack, G. R. 1994. Fatty acid synthesis: A potential selective target for antineoplastic therapy. Proc Natl Acad Sci USA 91: 6379–6383.
Kuhajda, F. P. 2000. Fatty-acid synthase and human cancer: New perspectives on its role in tumor biology. Nutrition 16: 202–208.
Kuhajda, F. P., Pizer, E. S., Li, J. N., Mani, N. S., Frehywot, G. L. and Townsend, C. A. 2000. Synthesis and antitumor activity of an inhibitor of fatty acid synthase. Proc Natl Acad Sci USA 97: 3450–3454.
Kuhajda, F. P. 2006. Fatty acid synthase and cancer: New application of an old pathway. Cancer Res 66: 5977–5980.
Kumar-Sinha, C., Ignatoski, K. W., Lippman, M. E., Ethier, S. P. and Chinnaiyan, A. M. 2003. Transcriptome analysis of HER2 reveals a molecular connection to fatty acid synthesis. Cancer Res 63: 132–139.
Lawrence, D. S., Zilfou, J. T. and Smith, C. D. 1999. Structure-activity studies of cerulenin analogues as protein palmitoylation inhibitors. J Med Chem 42: 4932–4941.
Li, B. H. and Tian, W. X. 2004. Inhibitory effects of flavonoids on animal fatty acid synthase. J Biochem (Tokyo) 135: 85–91.
Li, J. N., Mahmoud, M. A., Han, W. F., Ripple, M. and Pizer, E. S. 2000. Sterol regulatory element-binding protein-1 participates in the regulation of fatty acid synthase expression in colorectal neoplasia. Exp Cell Res 261: 159–165.
Li, J. N., Gorospe, M., Chrest, F. J., Kumaravel, T. S., Evans, M. K., Han, W. F. and Pizer, E. S. 2001. Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53. Cancer Res 61: 1493–1499.
Little, J. L., Wheeler, F. B., Fels, D. R., Koumenis, C. and Kridel, S. J. 2007. Inhibition of fatty acid synthase induces endoplasmic reticulum stress in tumor cells. Cancer Res 67: 1262–1269.
Liu, B., Wang, Y., Fillgrove, K. L. and Anderson, V. E. 2002. Triclosan inhibits enoyl-reductase of type I fatty acid synthase in vitro and is cytotoxic to MCF-7 and SKBr-3 breast cancer cells. Cancer Chemother Pharmacol 49: 187–193.
Loftus, T. M., Jaworsky, D. E., Frehywot, G. L., Townsend, C. A., Ronnett, G. V., Lane, M. D. and Kuhajda, F. P. 2000. Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors. Science 288: 2379–2381.
Lu, S. and Archer, M. C. 2005. Fatty acid synthase is a potential molecular target for the chemoprevention of breast cancer. Carcinogenesis 26: 153–157.
Magnard, C., Bachelier, R., Vincent, A., Jaquinod, M., Kieffer, S., Lenoir, G. M. and Venezia, N. D. 2002. BRCA1 interacts with acetyl-CoA carboxylase through its tandem of BRCT domains. Oncogene 21: 6729–6739.
Malvoisin, E. and Wild, F. 1990. Effect of drugs which inhibit cholesterol synthesis on syncytia formation in vero cells infected with measles virus. Biochim Biophys Acta 1042: 359–364.

Mathupala, S. P., Ko, Y. H. and Pedersen, P. L. 2006. Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. Oncogene 25: 4777–4786.

Medes, G., Thomas, A. and Weinhouse, S. 1953. Metabolism of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro. Cancer Res 13: 27–29.

Menendez, J. A., Mehmi, I., Atlas, E., Colomer, R. and Lupu, R. 2004a. Novel signaling molecules implicated in tumor-associated fatty acid synthase-dependent breast cancer cell proliferation and survival: Role of exogenous dietary fatty acids, p53-p21WAF1/CIP1, ERK1/2 MAPK, p27KIP1, BRCA1, and NF-kappaB. Int J Oncol 24: 591–608.

Menendez, J. A., Mehmi, I., Verma, V. A., Teng, P. K. and Lupu, R. 2004b. Pharmacological inhibition of fatty acid synthase (FAS): A novel therapeutic approach for breast cancer chemoprevention through its ability to suppress Her-2/neu (erbB-2) oncogene-induced malignant transformation. Mol Carcinog 41: 164–178.

Menendez, J. A., Vellon, L., Mehmi, I., Oza, B. P., Ropero, S., Colomer, R. and Lupu, R. 2004c. Inhibition of fatty acid synthase (FAS) suppresses HER2/neu (erbB-2) oncogene overexpression in cancer cells. Proc Natl Acad Sci USA 101: 10715–10720.

Menendez, J. A., Vellon, L., Colomer, R. and Lupu, R. 2005a. Pharmacological and small interference RNA-mediated inhibition of breast cancer-associated fatty acid synthase (oncogenic antigen-519) synergistically enhances Taxol (paclitaxel)-induced cytotoxicity. Int J Cancer 115: 19–35.

Menendez, J. A., Vellon, L. and Lupu, R. 2005b. Antitumoral actions of the anti-obesity drug orlistat (XenicalTM) in breast cancer cells: blockade of cell cycle progression, promotion of apoptotic cell death and PEA3-mediated transcriptional repression of Her2/neu (erbB-2) oncogene. Ann Oncol 16: 1253–1267.

Middleton, E., Jr., Kandaswami, C. and Theoharides, T. C. 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 52: 673–751.

Milgraum, L. Z., Witters, L. A., Pasternack, G. R. and Kuhajda, F. P. 1997. Enzymes of the fatty acid synthesis pathway are highly expressed in in situ breast carcinoma. Clin Cancer Res 3: 2115–2120.

Moncur, J. T., Park, J. P., Memoli, V. A., Mohandas, T. K. and Kinlaw, W. B. 1998. The "Spot 14" gene resides on the telomeric end of the 11q13 amplicon and is expressed in lipogenic breast cancers: implications for control of tumor metabolism. Proc Natl Acad Sci USA 95: 6989–6994.

Moore, S., Knudsen, B., True, L. D., Hawley, S., Etzioni, R., Wade, C., Gifford, D., Coleman, I. and Nelson, P. S. 2005. Loss of stearoyl-CoA desaturase expression is a frequent event in prostate carcinoma. Int J Cancer 114: 563–571.

Moreau, K., Dizin, E., Ray, H., Luquain, C., Lefai, E., Foufelle, F., Billaud, M., Lenoir, G. M. and Venezia, N. D. 2006. BRCA1 affects lipid synthesis through its interaction with acetyl-CoA carboxylase. J Biol Chem 281: 3172–3181.

Muck, A. O., Seeger, H. and Wallwiener, D. 2004. Inhibitory effect of statins on the proliferation of human breast cancer cells. Int J Clin Pharmacol Ther 42: 695–700.

Mycielska, M. E., Broke-Smith, T. P., Palmer, C. P., Beckerman, R., Nastos, T., Erguler, K. and Djamgoz, M. B. 2006. Citrate enhances in vitro metastatic behaviours of PC-3M human prostate cancer cells: status of endogenous citrate and dependence on aconitase and fatty acid synthase. Int J Biochem Cell Biol 38: 1766–1777.

Ohashi, K., Osuga, J., Tozawa, R., Kitamine, T., Yagyu, H., Sekiya, M., Tomita, S., Okazaki, H., Tamura, Y., Yahagi, N., Iizuka, Y., Harada, K., Gotoda, T., Shimano, H., Yamada, N. and Ishibashi, S. 2003. Early embryonic lethality caused by targeted disruption of the 3-hydroxy-3-methylglutaryl-CoA reductase gene. J Biol Chem 278: 42936–42941.

Omura, S. 1976. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol Rev 40: 681–697.

Ookhtens, M., Kannan, R., Lyon, I. and Baker, N. 1984. Liver and adipose tissue contributions to newly formed fatty acids in an ascites tumor. Am J Physiol 247: R146–153.

Osaki, M., Oshimura, M. and Ito, H. 2004. PI3K-Akt pathway: Its functions and alterations in human cancer. Apoptosis 9: 667–676.

Piyathilake, C. J., Frost, A. R., Manne, U., Bell, W. C., Weiss, H., Heimburger, D. C. and Grizzle, W. E. 2000. The expression of fatty acid synthase (FASE) is an early event in the development and progression of squamous cell carcinoma of the lung. Hum Pathol 31: 1068–1073.

Pizer, E. S., Jackisch, C., Wood, F. D., Pasternack, G. R., Davidson, N. E. and Kuhajda, F. P. 1996a. Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells. Cancer Res 56: 2745–2747.

Pizer, E. S., Wood, F. D., Heine, H. S., Romantsev, F. E., Pasternack, G. R. and Kuhajda, F. P. 1996b. Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer. Cancer Res 56: 1189–1193.

Pizer, E. S., Wood, F. D., Pasternack, G. R. and Kuhajda, F. P. 1996c. Fatty acid synthase (FAS): A target for cytotoxic antimetabolites in HL60 promyelocytic leukemia cells. Cancer Res 56: 745–751.

Pizer, E. S., Chrest, F. J., DiGiuseppe, J. A. and Han, W. F. 1998. Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines. Cancer Res 58: 4611–4615.

Pizer, E. S., Thupari, J., Han, W. F., Pinn, M. L., Chrest, F. J., Frehywot, G. L., Townsend, C. A. and Kuhajda, F. P. 2000. Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts. Cancer Res 60: 213–218.

Porstmann, T., Griffiths, B., Chung, Y. L., Delpuech, O., Griffiths, J. R., Downward, J. and Schulze, A. 2005. PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. Oncogene 24: 6465–6481.

Prowatke, I., Devens, F., Benner, A., Grone, E. F., Mertens, D., Grone, H. J., Lichter, P. and Joos, S. 2007. Expression analysis of imbalanced genes in prostate carcinoma using tissue microarrays. Br J Cancer 96: 82–88.

Rakheja, D., Kapur, P., Hoang, M. P., Roy, L. C. and Bennett, M. J. 2005. Increased ratio of saturated to unsaturated C18 fatty acids in colonic adenocarcinoma: Implications for cryotherapy and lipid raft function. Med Hypotheses 65: 1120–1123.

Ramirez de Molina, A., Rodriguez-Gonzalez, A., Gutierrez, R., Martinez-Pineiro, L., Sanchez, J., Bonilla, F., Rosell, R. and Lacal, J. 2002. Overexpression of choline kinase is a frequent feature in human tumor-derived cell lines and in lung, prostate, and colorectal human cancers. Biochem Biophys Res Commun 296: 580–583.

Rodriguez-Gonzalez, A., Ramirez de Molina, A., Banez-Coronel, M., Megias, D. and Lacal, J. C. 2005. Inhibition of choline kinase renders a highly selective cytotoxic effect in tumour cells through a mitochondrial independent mechanism. Int J Oncol 26: 999–1008.

Sabine, J. R., Abraham, S. and Chaikoff, I. L. 1967. Control of lipid metabolism in hepatomas: insensitivity of rate of fatty acid and cholesterol synthesis by mouse hepatoma BW7756 to fasting and to feedback control. Cancer Res 27: 793–799.

Sato, R. and Takano, T. 1995. Regulation of intracellular cholesterol metabolism. Cell Struct Funct 20: 421–427.

Sebastiani, V., Botti, C., Di Tondo, U., Visca, P., Pizzuti, L., Santeusanio, G. and Alo, P. L. 2006. Tissue microarray analysis of FAS, Bcl-2, Bcl-x, ER, PgR, Hsp60, p53 and Her2-neu in breast carcinoma. Anticancer Res 26: 2983–2987.

Sebti, S. M. 2005. Protein farnesylation: Implications for normal physiology, malignant transformation, and cancer therapy. Cancer Cell 7: 297–300.

Shah, U. S., Dhir, R., Gollin, S. M., Chandran, U. R., Lewis, D., Acquafondata, M. and Pflug, B. R. 2006. Fatty acid synthase gene overexpression and copy number gain in prostate adenocarcinoma. Hum Pathol 37: 401–409.

Shurbaji, M. S., Kalbfleisch, J. H. and Thurmond, T. S. 1996. Immunohistochemical detection of a fatty acid synthase (OA-519) as a predictor of progression of prostate cancer. Hum Pathol 27: 917–921.

Simons, K. and Ikonen, E. 1997. Functional rafts in cell membranes. Nature 387: 569–572.

Simons, K. and Toomre, D. 2000. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1: 31–39.

Swinnen, J. V., Ulrix, W., Heyns, W. and Verhoeven, G. 1997. Coordinate regulation of lipogenic gene expression by androgens: Evidence for a cascade mechanism involving sterol regulatory element binding proteins. Proc Natl Acad Sci USA 94: 12975–12980.

Swinnen, J. V., Heemers, H., Deboel, L., Foufelle, F., Heyns, W. and Verhoeven, G. 2000a. Stimulation of tumor-associated fatty acid synthase expression by growth factor activation of the sterol regulatory element-binding protein pathway. Oncogene 19: 5173–5181.

Swinnen, J. V., Vanderhoydonc, F., Elgamal, A. A., Eelen, M., Vercaeren, I., Joniau, S., Van Poppel, H., Baert, L., Goossens, K., Heyns, W. and Verhoeven, G. 2000b. Selective activation of the fatty acid synthesis pathway in human prostate cancer. Int J Cancer 88: 176–179.

Swinnen, J. V., Roskams, T., Joniau, S., Van Poppel, H., Oyen, R., Baert, L., Heyns, W. and Verhoeven, G. 2002. Overexpression of fatty acid synthase is an early and common event in the development of prostate cancer. Int J Cancer 98: 19–22.

Swinnen, J. V., Van Veldhoven, P. P., Timmermans, L., De Schrijver, E., Brusselmans, K., Vanderhoydonc, F., Van de Sande, T., Heemers, H., Heyns, W. and Verhoeven, G. 2003. Fatty acid synthase drives the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains. Biochem Biophys Res Commun 302: 898–903.

Swinnen, J. V., Heemers, H., van de Sande, T., de Schrijver, E., Brusselmans, K., Heyns, W. and Verhoeven, G. 2004. Androgens, lipogenesis and prostate cancer. J Steroid Biochem Mol Biol 92: 273–279.

Swinnen, J. V., Brusselmans, K. and Verhoeven, G. 2006. Increased lipogenesis in cancer cells: New players, novel targets. Curr Opin Clin Nutr Metab Care 9: 358–365.

Szutowicz, A., Kwiatkowski, J. and Angielski, S. 1979. Lipogenetic and glycolytic enzyme activities in carcinoma and nonmalignant diseases of the human breast. Br J Cancer 39: 681–687.

Takahashi, K. A., Smart, J. L., Liu, H. and Cone, R. D. 2004. The anorexigenic fatty acid synthase inhibitor, C75, is a nonspecific neuronal activator. Endocrinology 145: 184–193.

Thupari, J. N., Pinn, M. L. and Kuhajda, F. P. 2001. Fatty acid synthase inhibition in human breast cancer cells leads to malonyl-CoA-induced inhibition of fatty acid oxidation and cytotoxicity. Biochem Biophys Res Commun 285: 217–223.

Thupari, J. N., Landree, L. E., Ronnett, G. V. and Kuhajda, F. P. 2002. C75 increases peripheral energy utilization and fatty acid oxidation in diet-induced obesity. Proc Natl Acad Sci USA 99: 9498–9502.

Tozawa, R., Ishibashi, S., Osuga, J., Yagyu, H., Oka, T., Chen, Z., Ohashi, K., Perrey, S., Shionoiri, F., Yahagi, N., Harada, K., Gotoda, T., Yazaki, Y. and Yamada, N. 1999. Embryonic lethality and defective neural tube closure in mice lacking squalene synthase. J Biol Chem 274: 30843–30848.

Turyn, J., Schlichtholz, B., Dettlaff-Pokora, A., Presler, M., Goyke, E., Matuszewski, M., Kmiec, Z., Krajka, K. and Swierczynski, J. 2003. Increased activity of glycerol 3-phosphate dehydrogenase and other lipogenic enzymes in human bladder cancer. Horm Metab Res 35: 565–569.

Van de Sande, T., De Schrijver, E., Heyns, W., Verhoeven, G. and Swinnen, J. V. 2002. Role of the phosphatidylinositol 3 -kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells. Cancer Res 62: 642–646.

Van de Sande, T., Roskams, T., Lerut, E., Joniau, S., Van Poppel, H., Verhoeven, G. and Swinnen, J. V. 2005. High-level expression of fatty acid synthase in human prostate cancer tissues is linked to activation and nuclear localization of Akt/PKB. J Pathol 206: 214–219.

van der Sanden, M. H., Houweling, M., van Golde, L. M. and Vaandrager, A. B. 2003. Inhibition of phosphatidylcholine synthesis induces expression of the endoplasmic reticulum stress and

apoptosis-related protein CCAAT/enhancer-binding protein-homologous protein (CHOP/GADD153). Biochem J 369: 643–650.
Veillard, N. R. and Mach, F. 2002. Statins: The new aspirin? Cell Mol Life Sci 59: 1771–1786.
Visca, P., Sebastiani, V., Botti, C., Diodoro, M. G., Lasagni, R. P., Romagnoli, F., Brenna, A., De Joannon, B. C., Donnorso, R. P., Lombardi, G. and Alo, P. L. 2004. Fatty acid synthase (FAS) is a marker of increased risk of recurrence in lung carcinoma. Anticancer Res 24: 4169–4173.
Wang, H. Q., Altomare, D. A., Skele, K. L., Poulikakos, P. I., Kuhajda, F. P., Di Cristofano, A. and Testa, J. R. 2005. Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells. Oncogene 24: 3574–3582.
Wang, X. and Tian, W. 2001. Green tea epigallocatechin gallate: A natural inhibitor of fatty-acid synthase. Biochem Biophys Res Commun 288: 1200–1206.
Weiss, L., Hoffmann, G. E., Schreiber, R., Andres, H., Fuchs, E., Korber, E. and Kolb, H. J. 1986. Fatty-acid biosynthesis in man, a pathway of minor importance. Purification, optimal assay conditions, and organ distribution of fatty-acid synthase. Biol Chem Hoppe Seyler 367: 905–912.
Wieman, H. L., Wofford, J. A. and Rathmell, J. C. 2007. Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. Mol Biol Cell 18: 1437–1446.
Xia, Z., Tan, M. M., Wong, W. W., Dimitroulakos, J., Minden, M. D. and Penn, L. Z. 2001. Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia 15: 1398–1407.
Yahagi, N., Shimano, H., Hasegawa, K., Ohashi, K., Matsuzaka, T., Najima, Y., Sekiya, M., Tomita, S., Okazaki, H., Tamura, Y., Iizuka, Y., Nagai, R., Ishibashi, S., Kadowaki, T., Makuuchi, M., Ohnishi, S., Osuga, J. and Yamada, N. 2005. Co-ordinate activation of lipogenic enzymes in hepatocellular carcinoma. Eur J Cancer 41: 1316–1322.
Yang, C. S., Landau, J. M., Huang, M. T. and Newmark, H. L. 2001. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu Rev Nutr 21: 381–406.
Yang, Y. A., Han, W. F., Morin, P. J., Chrest, F. J. and Pizer, E. S. 2002. Activation of fatty acid synthesis during neoplastic transformation: Role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Exp Cell Res 279: 80–90.
Yeh, C. W., Chen, W. J., Chiang, C. T., Lin-Shiau, S. Y. and Lin, J. K. 2003. Suppression of fatty acid synthase in MCF-7 breast cancer cells by tea and tea polyphenols: A possible mechanism for their hypolipidemic effects. Pharmacogenomics J 3: 267–276.
Yoon, S., Lee, M. Y., Park, S. W., Moon, J. S., Koh, Y. K., Ahn, Y. H., Park, B. W. and Kim, K. S. 2007. Up-regulation of acetyl-CoA carboxylase {alpha} and fatty acid synthase by human epidermal growth factor receptor 2 at the translational level in breast cancer cells. J Biol Chem 282: 26122–26131.
Zhao, W., Kridel, S., Thorburn, A., Kooshki, M., Little, J., Hebbar, S. and Robbins, M. 2006. Fatty acid synthase: A novel target for antiglioma therapy. Br J Cancer 95: 869–878.
Zhong, W. B., Wang, C. Y., Chang, T. C. and Lee, W. S. 2003. Lovastatin induces apoptosis of anaplastic thyroid cancer cells via inhibition of protein geranylgeranylation and de novo protein synthesis. Endocrinology 144: 3852–3859.
Zhou, W., Simpson, P. J., McFadden, J. M., Townsend, C. A., Medghalchi, S. M., Vadlamudi, A., Pinn, M. L., Ronnett, G. V. and Kuhajda, F. P. 2003. Fatty acid synthase inhibition triggers apoptosis during S phase in human cancer cells. Cancer Res 63: 7330–7337.
Zhou, W., Han, W. F., Landree, L. E., Thupari, J. N., Pinn, M. L., Bililign, T., Kim, E. K., Vadlamudi, A., Medghalchi, S. M., El Meskini, R., Ronnett, G. V., Townsend, C. A. and Kuhajda, F. P. 2007. Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells. Cancer Res 67: 2964–2971.
Zhuang, L., Lin, J., Lu, M. L., Solomon, K. R. and Freeman, M. R. 2002. Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. Cancer Res 62: 2227–2231.
Zhuang, L., Kim, J., Adam, R. M., Solomon, K. R. and Freeman, M. R. 2005. Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. J Clin Invest 115: 959–968.

Citrate Metabolism in Prostate and Other Cancers

Renty B. Franklin and Leslie C. Costello

The Metabolic Roles of Citrate in Normal Mammalian Cells

Citrate occupies a critical crossroad step in the intermediary metabolism of most normal mammalian cells (Fig. 1). It is synthesized in the mitochondria, where it becomes the entry oxidative substrate into the Krebs cycle. As such, its oxidation provides the major source of cellular ATP production. Because citrate is the common intermediate that results from glucose and fatty acid utilization, it is the link for their complete oxidation. This is the bioenergetic role of citrate that is most commonly considered to be the major relationship of citrate in cellular intermediary metabolism. However citrate serves other important metabolic roles. By virtue of its oxidation via the Krebs cycle, it provides several intermediates that couples the Krebs cycle with other associated synthetic and catabolic pathways of metabolism. For example, its oxidation to alpha ketoglutarate can be coupled by aminotransferases for the synthesis of amino acids such as aspartate. Another critical role of citrate is in relation to cells that are engaged in lipogenesis and cholesterogenesis. In this case, citrate is shuttled out of the mitochondria and converted in the cytosol to acetyl CoA, which is the essential precursor for de novo lipogenesis/cholesterogenesis. Consequently, citrate metabolism is a central and essential component in the intermediary energy metabolism of normal mammalian cells. However, there is one notable exception. As will be discussed later, in normal prostate epithelial cells, citrate is a secretory end-product of metabolism rather than a utilized substrate of intermediary metabolism.

The following reactions of the cellular energy production derived from glucose oxidation illustrate the important bioenergetic role of citrate oxidation.

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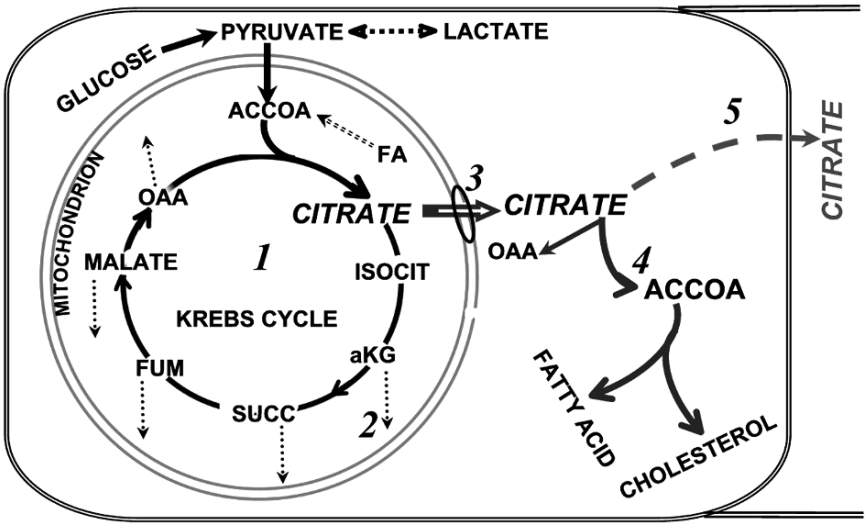


Fig. 1 The pathways of citrate utilization. Citrate is synthesized in the mitochondria from acetyl CoA + oxalacetate. Citrate enters the Krebs cycle, where it is oxidized (1) and/or provides intermediates that are utilized in associated pathways (2). Citrate is exported from the mitochondria to the cytosol (3), where it is converted to acetyl CoA (4) for lipogenesis/cholesterogenesis, or the citrate is secreted from the cell (5) as in prostate cells

(1) Anoxic Glycolytic Pathway:

$$\mathrm{GLUC} \longrightarrow 2\ \mathrm{LACT} + 2\ \mathrm{ATP}$$

(2) Normoxic Glycolytic pathway:

$$\mathrm{GLUC} + 6\ \mathrm{O2} \longrightarrow 2\ \mathrm{PYR} + 8\ \mathrm{ATP} \longrightarrow 6\ \mathrm{CO2} + 30\ \mathrm{ATP} \Big\} 38\ \mathrm{ATP}$$

(3) Oxidation of citrate

$$2\ \mathrm{CIT} + 4\ \mathrm{O2} \longrightarrow 2\ \mathrm{OAA} + 4\ \mathrm{CO2} + 24\ \mathrm{ATP}$$

We have represented glycolysis under anaerobic conditions as the "anoxic glycolytic pathway" (reaction 1), which we believe is a more physiological relevant terminology. This is also the pathway that is characterized as aerobic glycolysis as described for tumor cell metabolism. Typically in most normal mammalian cells under aerobic conditions, the complete oxidation of glucose (reaction 2) results in the production of 38 ATP/glucose oxidized, which includes 6 ATP from the oxidation of glycolytic-produced NADH. Of this amount, the oxidation of citrate

results in 24 ATP produced, which is ~63% of the total ATP produced from the complete oxidation of glucose. Therefore when citrate is diverted from its complete oxidation via the Krebs cycle, a bioenergetic cost is imposed upon the cell. The export and utilization of citrate for cellular lipogenesis/cholesterogenesis or for citrate secretion by normal prostate cells results in the loss of 12 ATP/citrate or 24 ATP/glucose utilized, i.e., a 63% loss of potential ATP production.

De novo lipogenesis and cholesterogenesis occurs in the cytosol and requires a source of acetyl CoA as the building block for fatty acid synthesis (8 acetyl CoA/palmitate) and for lanesterol synthesis (12 acetyl CoA/lanesterol). Most normal functionally differentiated mammalian cells are not engaged in this metabolic activity. The exceptions are specialized lipogenic and cholesterogenic cells, normal cells that are proliferating/regenerating, and proliferating malignant cells. Generally, the cytosolic acetyl CoA is derived from the mitochondrial citrate pool. Under normal conditions most mammalian cells retain citrate within the mitochondrial compartment for its utilization via the Krebs cycle. An up regulation of the citrate transporter (CTP) must occur for any significant portion of the intramitochondrial pool of citrate to be exported to the cytosol. Correspondingly, an up regulation of ATP citrate lyase must occur to convert the cytosolic citrate to acetyl CoA. There is some evidence that the cytosolic citrate might also be oxidized via cytosolic NADP-isocitrate dehydrogenase to recycle NADPH as a reducing factor in cytosolic metabolic pathways.

Axioms of Relationships of Cellular Activity, Cellular Metabolism, and Malignancy

Before proceeding further, some important generalizations that we consider to be axiomatic should be considered.

1. In all cells, the existing cellular intermediary metabolism provides the bioenergetic/synthetic/catabolic requirements that are essential for the manifestation of the cells' current activities (function, growth, proliferation).
2. When a cell's activity changes, its metabolism must also be adjusted consistent with any newly established bioenergetic/synthetic/catabolic requirements.
3. Malignant cells exhibit a parasitic existence. They have no specialized function other than the activities essential for their generational propagation (growth and proliferation), which occurs at the expense of their host.
4. Malignant cells are derived from normal cells that have undergone a genetic transformation to a neoplastic cell phenotype that is endowed with malignant potential.
5. Manifestation of the malignant potential of the neoplastic cell necessitates alterations in its metabolism (i.e., a metabolic transformation) to provide the bioenergetic/synthetic requirements of malignancy.

6. In the absence of the metabolic transformation, the neoplastic cell will not progress to complete malignancy. Conversely, the metabolic transformation, in the absence of the genetic transformation to a neoplastic malignant cell, will not cause malignancy.
7. Common to all malignant cells is the metabolic requirement for de novo lipogenesis/cholesterogenesis for membraneogenesis that is essential for their proliferative existence.

Citrate Metabolism of Normal Prostate Epithelial Cells: Net Citrate Production

For detailed description and reviews of the relationships of citrate metabolism in normal prostate and in prostate cancer, we refer the reader to our recent reports (Costello and Franklin 2001, 2002, 2006; Costello et al. 1999, 2005; Franklin and Costello 2007; Franklin et al. 2005). The human prostate gland is a complex organ composed of different zones that have different embryological origin and different functional activities. The focus of this presentation is the peripheral zone, which comprises about 70% of the gland, and is the major functional component. Most importantly, this is the major region of malignancy. The primary function of the prostate gland is the production and secretion of prostatic fluid. The major component of the prostatic fluid is its extraordinarily high concentration of citrate, which ranges from ~40–150 mM as contrasted with ~0.2 mM citrate in blood plasma (Table 1). Correspondingly, normal peripheral zone tissue citrate levels range from ~12,000–14,000 nmol/g as contrasted with other tissues that generally contain ~250–450 nmol/g.

The function of prostate citrate production is achieved by the activity of highly specialized glandular epithelial cells that have evolved for the capability to accumulate and secrete citrate, which we refer to as "net citrate production". This capability does not exist in other mammalian cells. Consequently the intermediary metabolism of these "citrate-producing" cells is "driven and modified" by their specialized function of net citrate production (Fig. 2). In typical mammalian cell intermediary metabolism, glucose utilization provides the acetyl CoA that condenses with OAA to produce citrate, which is oxidized via the Krebs cycle with the

Table 1 Representative prostate levels

	Citrate (nmol/g wwt)	Zinc (nmol/g wwt)
Normal peripheral zone	12,000–14,000	3,000–4,500
PCA malignant tissue	200–2,000	400–800
Other tissues	250–450	200–400
Normal prostatic fluid	40,000–150,000	8,000–10,000
PCA prostatic fluid	1,000–30,000	250–1,000
Blood plasma	100–200	15

regeneration of OAA. Consequently the oxidation of six-carbon citrate results in the loss of two carbons via CO_2 production, and four carbons are conserved as OAA. In the citrate-producing prostate cells, all six carbons are removed from the metabolic pool as an end product of metabolism. Therefore, citrate synthesis in these cells requires the availability of a source of acetyl CoA and a source of OAA. The former is derived from glucose, and the latter is derived from aspartate. One must recognize the magnitude of prostate citrate production. The production of 1 mL of prostatic fluid, on average, involves the production of 100 μmols citrate. To achieve this, 50 μmols glucose and 100 μmols aspartate will be utilized. The concentration of glucose and aspartate in blood plasma is 5 μmols/mL and 0.03 μmols/mL, respectively. Thus, a tremendous demand on the availability of glucose and aspartate is required for prostate citrate production.

To meet the precursor requirements for continued citrate synthesis, the prostate epithelial cells possess unique glucose and aspartate relationships. These cells, unlike most other mammalian cells, utilize glucose via a high aerobic glycolysis (Huggins 1947; Harkonen 1981; Costello and Franklin 1989) that provides a high production of lactate← →pyruvate that is available for the production of acetyl coA. For the production of OAA, aspartate is accumulated in the prostate cells and is transaminated via mAAT and coupled to GDH activity in a pathway that we define as the aspartate–glutamate–citrate pathway (Fig. 2). In these cells aspartate is an essential amino acid that is utilized, which is unlike most other mammalian cells that synthesize aspartate as a nonessential amino acid. Because the plasma aspartate concentration is low (0.03 mM) relative to its requirement for high citrate production, the prostate cells maintain a high cellular aspartate concentration (1.2 mM) availability for citrate synthesis (Franklin, Lao, and Costello 1990). To achieve this, the prostate cells exhibit a high-affinity L-aspartate transport process that was recently identified as EAAC1 (Lao et al. 1993; Costello et al. 1993; Franklin et al. 2006). In other mammalian cells EAAC1 generally operates as a high-affinity glutamate transporter rather than an aspartate transporter.

The relationships described earlier involve the metabolic adaptations that are essential to facilitate citrate synthesis. The accumulation of citrate is dependent upon its rate of synthesis vs. its rate of utilization. The prevention of citrate oxidation by the prostate cells is the key relationship responsible for net citrate production. The prostate peripheral zone accumulates extremely high levels of zinc in the range of ~3,000–5,000 nmol/g when compared with other tissues being ~200–400 nmol/g. The cellular accumulation of zinc results in high levels of mitochondrial zinc, which inhibits m-aconitase activity and citrate oxidation (Costello et al. 1997). That zinc inhibition is the essential cause of limiting m-aconitase activity is evident from the recent observation that the level of m-aconitase enzyme is not low or changed in normal or malignant human and animal prostate cells (Singh et al. 2006), The inhibition of m-aconitase truncates the Krebs cycle at the first step of citrate oxidation, which provides the most efficient metabolic alteration for synthesized citrate to accumulate for secretion (Fig. 2). As described earlier, to accumulate citrate for secretion these specialized cells sacrifice ~60% of the potential energy that could be derived from complete oxidation of glucose.

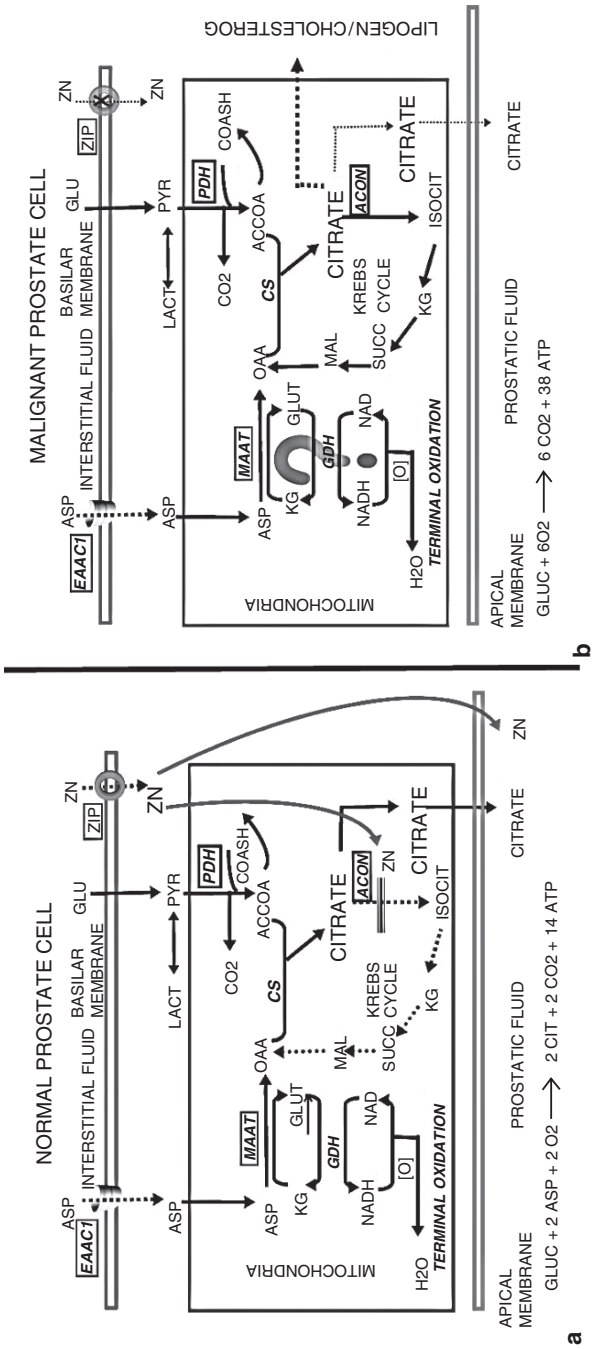


Fig. 2 The pathway of net citrate production in normal prostate cells (**a**) vs. the pathway of citrate utilization in malignant prostate cells (**b**) The key step in the pathway to net citrate production in normal cells is the expression of Zip1 resulting in zinc accumulation, which inhibits m-aconitase and citrate oxidation. The key step in the metabolic transformation is the silencing of Zip1 expression that eliminates the accumulation of zinc and its inhibition of m-aconitase and citrate oxidation via the Krebs cycle in the malignant cells

In the absence of any reported studies, one must presume that the normal prostate cells possess an active citrate shuttle process (presumably citrate transporter protein, CTP) that permits the export of most of the mitochondrial citrate pool into the cytosol and finally secreted into prostatic fluid. This also implies the likelihood that ATP-citrate lyase activity is low or absent to conserve the cytosolic citrate for secretion, especially since the prostate epithelial cells have no significant demand for de novo lipogenesis/cholesterogenesis. Halliday et al. (1988) did infer that ATP citrate lyase activity was low in benign prostate glands and increased in malignant prostate glands, but direct studies of the enzyme were not conducted.

Altered Citrate Relationships in Prostate Cancer: The Metabolic Transformation

It is now well established that citrate and zinc levels are markedly decreased in malignant vs. normal prostate tissue. These biochemical changes occur early in the development of malignancy. Moreover, malignant prostate foci virtually never retain the characteristic high levels of citrate and zinc found in normal peripheral zone. In the absence of high cellular zinc levels, m-aconitase activity is no longer inhibited and citrate oxidation proceeds via the Krebs cycle (Fig. 2). Thus we characterize the metabolic transformation as the transformation of zinc-accumulating citrate-producing normal prostate epithelial cells to citrate-oxidizing cells that have lost the ability to accumulate zinc. An important benefit of this metabolic transformation is the bioenergetic relationship in that citrate oxidation provides the additional 24 ATP from complete glucose oxidation that were lost by incomplete glucose oxidation in net citrate production. Thus, the malignant cells are bioenergetically more efficient than the specialized normal prostate epithelial cells. This is in contrast to the general tumor metabolism relationship in which the tumor cells are metabolically transformed to high aerobic glycolysis (Warburg et al. 1926; Racker and Spector 1981; Bagetto 1992; Costello and Franklin 2005). Figure 3 shows our concept of the genetic/metabolic transformation of the citrate-producing normal epithelial cell to the citrate-oxidizing malignant cell in the development of prostate malignancy.

The Metabolic Role of Citrate in Malignancy: Oxidation and/or Cytosolic Acetyl CoA Production

It would be misleading and inaccurate to presume that all of the mitochondrial citrate pool is destined for oxidation via the Krebs cycle. For the malignant cells to proliferate they must also adapt their metabolism for the utilization of citrate for de novo lipid biosynthesis. The initiating steps in this pathway are the export of citrate into cytosol followed by its conversion to acetyl CoA. Since the normal prostate

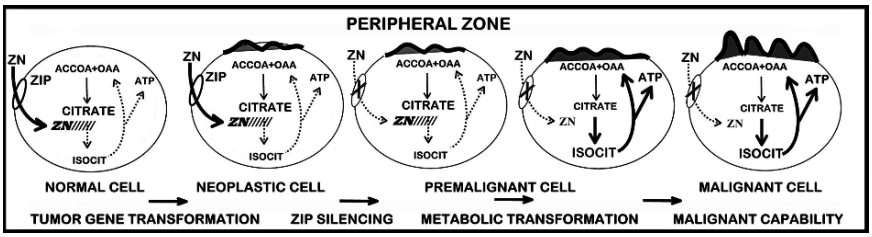


Fig. 3 The concept of the metabolic transformation in the development of malignant prostate cells. The zinc accumulating citrate-producing normal cell is genetically transformed to a neoplastic cell with malignant potential. Zip 1 expression is silenced in the neoplastic cell, which loses the ability to accumulate zinc as a premalignant stage. The depletion of zinc removes its inhibition of m-aconitase so that citrate oxidation via the Krebs cycle and coupled ATP production can occur. The citrate oxidizing malignant cell is now capable of fulfilling the bioenergetic, synthetic, and proliferative activities of malignancy

cells must posses the mechanism for citrate export to cytosol for secretion, the malignant cells would not require an upregulation of this process. However, an essential metabolic adaptation in the malignant prostate cells must be an increase in ATP citrate lyase activity that likely does not exist in the normal cells. Halliday, Fenoglio-Preiser and Sillerud (1988) observed that a decrease in citrate levels correlates with a corresponding increase fatty acid levels in prostate malignant glands as contrasted with no such changes in neighboring BPH glands. They concluded that this is indicative of an increase in ATP citrate lyase activity in malignancy. Swinnen et al. (2002) present an informative review of existing evidence that the expression and levels of some important lipogenic/cholesterogenic enzymes are upregulated in malignant prostate cells. It is of particular interest that FAS levels are increased in PIN and more so in the adenocarcinomatous glands, which is consistent with the metabolic transformation occurrence early in malignancy. It is apparent that the development and progression of prostate malignancy involves a metabolic shift to de novo lipid biosynthesis. The metabolic adaptation for citrate export and its conversion to cytosolic acetyl CoA would be applicable to all malignant cell types that are derived from normal nonlipogenic/noncholesterogenic cells, and could preexist in the normal lipogenic/cholesterogenic cells.

The consideration of the lipogenic/cholesterogenic requirement of malignant cells has lead to the concept of a "truncated Krebs cycle" as a prevailing view of tumor cell metabolism (Parlo and Coleman 1984, 1986). The concept proposes that an increased citrate shuttle activity causes rapid export of citrate, which depletes citrate for oxidation by the Krebs cycle. It is well documented that the Krebs cycle downstream reactions from isocitrate to oxalacetate are operational in tumor cells (for review see Costello and Franklin 2005). However, the critical reaction is m-aconitase about which little is known in tumor cells other than our studies with prostate cells. If cells posses m-aconitase activity and isocitrate oxidation, it is not likely that the rate of citrate export could deplete citrate so that essentially no citrate oxidation would occur. The kinetic relationships of citrate export vs. m-aconitase

activity dictate that m-aconitase activity and citrate oxidation will prevail in the presence of a sufficient intramitochondrial pool of citrate that would support high citrate export. Therefore, in prostate malignant cells, and likely in other tumor cells, citrate oxidation and citrate export can coexist as long as sufficient precursors are available for citrate synthesis.

There is an important metabolic distinction between the export of citrate for secretion as occurs in normal prostate epithelial cells and the export of citrate for cytosolic acetyl CoA production as occurs in lipogenic/cholesterogenic cell including proliferating tumor cells (Fig. 4). The export of citrate which occurs as a divalent anion alone with H+ requires the import of a counter ion. If the cytosolic citrate is converted to acetyl CoA + oxalacetate by ATP-citrate lyase, the oxalacetate can be reduced to malate by cMDH which serves as the counterion. The imported malate then provides the source of mitochondrial oxalacetate for citrate synthesis. In contrast, when citrate is secreted out of the cell, there is no regeneration of the counter ion for citrate export. Then some other source, yet to be identified, of an appropriate counterion must exist.

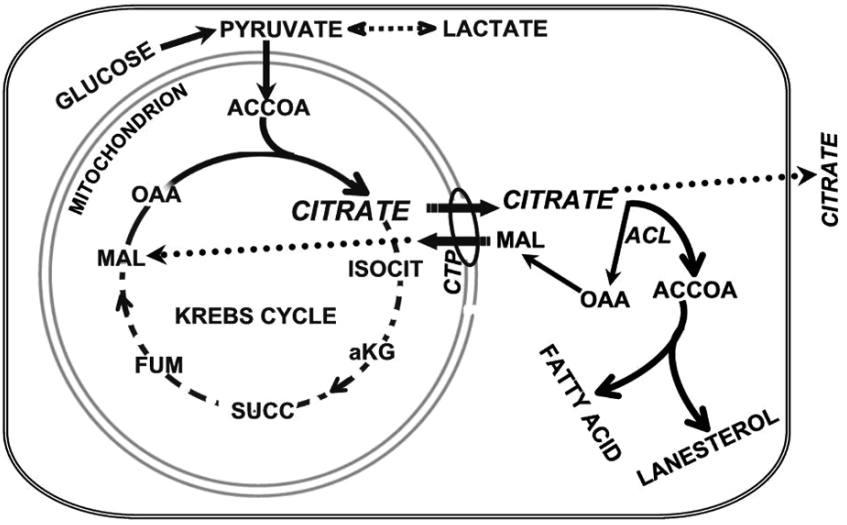


Fig. 4 The citrate shuttle process and the fate of cytosolic citrate. CTP (citrate transporter protein) exports citrate to cytosol. Cytosolic citrate is converted by ATO-citrate lyase to acetyl CoA + oxalacetate. The acetyl CoA is utilized for lipogenesis/cholesterogenesis. The oxalacetate is converted to malate by malic dehydrogenase, and malate is transported into the mitochondria as counterion for the export of citrate. The malate is converted to oxalacetate for the synthesis of citrate. In this process the four-carbon moiety is preserved. When the cytosolic citrate is secreted from the cell, all six carbons are lost. Then a counterion must be provided for citrate export to occur

The Role of Accelerated Glycolysis in Tumor Cell Metabolism

Eight decades have transpired since the hallmark studies of Warburg et al. (1926) that initiated the focus on altered intermediary metabolism of tumor cells. The focal observation was the shift in intermediary metabolism from the typical aerobic metabolism of the normal mammalian cells to the high level of "aerobic glycolysis" and low respiration that characterized many, but not all, tumor cells. One must also recognize that the population of tumor cells that comprise an existing and developing tumor in situ are not exposed to the same environmental conditions. For example, a gradient of perfusate and its composition that will exist in situ imposes a changing oxygen environment from normoxia to hypoxia to anoxia. There will also be a changing availability of perfusate glucose and other micronutrients that are derived from the host circulation via interstitial fluid. Such conditions impact the cellular metabolism of the tumor cells. Under in situ conditions (especially for solid tumors) the population of tumor cells is not likely to exhibit a uniform intermediary metabolism, but will exhibit varying metabolic relationships. In vitro studies of tumor cell metabolism often will not reflect the realities that exist in the in situ malignant process.

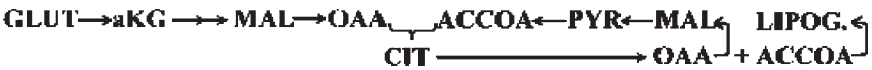
The importance of or reason for the transition to high aerobic glycolysis by tumor cells has been an intriguing question that has persisted over the past eighty years. The description of a "high aerobic glycolysis" in tumor cells implies the production of lactic acid + 2 ATP/glucose utilized, presumably to replace the lost ATP production due to defective oxidation via Krebs cycle/terminal oxidation/coupled phosphorylation. The tumor cell glucose utilization would have to increase 19-fold to achieve the same bioenergetic results of the normoxic glycolytic pathway and complete oxidation. This can be achieved since the glycolytic production of ATP occurs at a rate that is 100-times faster than the mitochondrial oxidation to CO_2 + ATP. The nature of the successful metabolic adaptation the tumor cells is demonstrated by their efficient extraction of glucose from and at the expense of the host. One must recognize that, in the absence of alternative substrate sources, this "anoxic" glycolytic pathway is incompatible with the de novo lipogenic/cholesterogenic requirements for tumor cell growth/proliferation. The high aerobic glycolysis would exist primarily for the purpose of providing a readily available source of ATP while the tumor cells are subjected to a physiological anoxic condition in which the availability of oxygen is insufficient for pyruvate oxidation to keep pace with glucose utilization by the cells to support the aerobic metabolism of the cells. This would be a stage in which the tumor cells are not proliferating.

In order to demonstrate the aforementioned condition, one must conduct a stoichiometric analysis of the glucose utilized and the amount of lactate produced, and demonstrate that essentially all of the utilized glucose is accounted for by lactate. Simply measuring the latter is insufficient. For the growth/proliferation of the tumor, the tumor cells must be provided with the substrate(s) for de novo lipogenesis/cholesterogenesis. This requires the availability of citrate for the production of cytosolic acetyl CoA, unless some other carbon source might be

available. The accelerated glycolysis of tumor cells will provide for the production of citrate from the oxidation of pyruvate as well as the additional production of ATP (as described earlier). This is also consistent with the conclusion of Pedersen (1978) that 60–85% of the ATP production by tumor cells is mitochondrial-generated. The fact that a considerable portion of the glucose utilized under normoxic/hypoxic conditions ends up as lactate cannot be ignored. As concluded by Pedersen (1978), the accumulating pyruvate is subjected to reduction to lactate. The oxidation of pyruvate for citrate synthesis might be further limited by the availability of OAA, which can be derived from the export of citrate. Until then, the excess pyruvate is reduced to lactic acid. This insures that a sufficient pool of pyruvate is available for lipogenesis. An appropriate stoichiometric analysis of overall glucose utilization by the combined pathways should be:

$$\text{glucose utilized} = (\text{lactate} + \text{citrate} + CO_2) + \text{other?};$$
$$\text{where (citrate)} = (\text{citrate conc}) + (\text{“lipogenic/cholesterogenic” AcCoA})$$

As Moreadith and Lehninger (1984) observed, “*. . . many malignant cell lines, as well as some normal cells, do not have an absolute requirement for glucose per se. . .*” Those tumor cells that do not “glycolyze” must posses an alternative pathway. They proposed that a glutamate pathway could provide both OAA and ACCOA for citrate production for lipogenesis, in the absence of glucose contribution. McKeechan (1982) proposed the utilization of glutaminolysis as a source of pyruvate in tumor cells. The following is a modified representation of the pathway of glutaminolysis through citrate to lipogenesis:



An important distinction between prostate tumor cell metabolism vs. other tumor cells is the transition to a high aerobic glycolysis, low respiration metabolic characteristic. Unlike other normal mammalian cells, the normal prostate epithelial cells are already high aerobic glycolytic, low respiring cells due to their function of net citrate production. As such, these cells are transformed to citrate utilizing malignant cells with increased citrate oxidation and citrate utilization for cytosolic acetyl CoA production. Other cells must undergo the transformation from normal oxidation of glucose to high aerobic glycolytic malignant cells.

Zinc and Mitochondrial Metabolism Relationships

Zinc plays a major role in the citrate metabolism of normal prostate cells and in the metabolic transformation in malignant cells. This necessitates an understanding of some important interrelationships of zinc with mitochondria. For this discussion we will define three pools of zinc that comprise the total intracellular zinc (for review

see Franklin et al. 2005): (1) tightly bound zinc (mainly metalloenzymes, metalloproteins, and nucleoproteins) that is an immobile unreactive pool; (2) loosely bound zinc (such as amino acid and citrate bound) that constitutes a mobile reactive pool; and (3) free Zn^{2+} ion that is a reactive pool. The total cellular zinc content of mammalian cells is estimated to approximate 0.2–1.0 mM of which the free Zn^{2+} ion pool (nM–fM range) is negligible (Krezel et al. 2007; Vallee and Falchuk, 1994; Outten and O'Halloran 2001). In the prostate, the cytosolic zinc approximates 0.6–3.0 mM. The mobile transportable ZnLigand pool approximates 20–100 mM Zn in most mammalian cells and about 60–300 mM in normal prostate cells. These relationships raise the question, "In the absence of the existence of a free Zn^{2+} ion pool, how is mobile reactive zinc incorporated into the cell and trafficked through the cytosol and into the mitochondria. The first step is the transport of zinc from circulation into the cell. This is achieved via plasma membrane zinc uptake transporters, which in normal prostate cells involves the Zip1 transporter (and perhaps other transporters). Relative to mitochondrial zinc, the next issue is the process by which the cytosolic zinc is delivered into the mitochondria. This important process has not been identified The outer mitochondrial membrane pore structure permits the passage of molecules up to ~10 kDa, so that ZnLigands of this size and smaller constitute the cytosolic pool that delivers zinc into the mitochondrial intermembrane space. The intermembranous pool of ZnLigands is available for interaction with mitochondrial components that reside in and have exposure in the intermembrane space, such as the electron transport components, and is also the pool of ZnLigands available for transport of zinc across the highly impermeable inner membrane and into the mitochondrial matrix. The mitochondria contain a putative zinc uptake transporter that is associated with the mitochondrial inner membrane (Guan et al. 2003). This unidentified transporter is capable of transporting zinc from mobile reactive cytosolic zinc complexes that have binding affinities up to log *Kf* ~12 (e.g. metallothionein, citrate, cysteine, aspartate, histidine) into the matrix compartment of the mitochondria.

Because prostate cells have a higher concentration of cellular zinc than other cells, this process results in a higher concentration of mitochondrial zinc. The high concentration of intramitochondrial zinc in normal prostate cells results in several effects on mitochondrial metabolism and function. Its effect on the inhibition of m-aconitase and citrate oxidation as has been described above. In addition, zinc has an inhibitory effect on the terminal oxidation and prostate mitochondria (Costello et al. 2004). Prostate had been characterized as a tissue that possesses a low respiration (Huggins 1947; Muntzing et al. 1975), which suggests the possibility that terminal oxidation might be a limiting factor that results in low oxygen consumption. The activities of components of complexes I–IV and respiration are markedly lower in prostate mitochondria than in other cells (Costello et al. 1976; Costello et al. 2004). This is due to two factors. The constitutive levels of activity of complexes I–IV of prostate mitochondria are markedly lower (50–80% lower) than liver mitochondria as is the respiration of the mitochondria. In addition, the high levels of zinc inhibit the respiration and terminal oxidation of prostate mitochondria, which appears to be due to an inhibition at complex III. The inhibition occurs also with isolated liver

mitochondria when supplemented with high zinc levels. Therefore the combination of low levels of electron transport components and the inhibitory effect of high zinc accumulation on terminal oxidation are major factors associated with the low respiration of the normal prostate cells. This, along with the inhibition of citrate oxidation, contributes to the inefficient mitochondrial coupled ATP production that characterizes the normal prostate cells, and that is compensated for by the characteristic high aerobic glycolysis.

These metabolic effects of zinc accumulation in normal prostate cells would have serious deleterious effects on function and survival of most other mammalian cells. We must also note that zinc accumulation also results in inhibition of proliferation of prostate cells due to zinc induction of mitochondrial apoptogenesis (Feng et al. 2000, 2002). However, the highly specialized prostate cells have evolved for the function of net citrate production and secretion, and the evolution of these cells has involved adaptive metabolic activities that permit their survival and function. The loss of the capability of zinc accumulation that accompanies the development and progression of prostate cancer ensures that the deleterious effects of zinc on metabolism and proliferation are not imposed upon the malignant cells. For this reason, malignant prostate cells in situ do not exist as zinc-accumulating cells. Therefore we have proposed that zinc is an tumor suppressor agent in prostate cancer. Since Zip1 is essential for zinc accumulation in prostate cells and is down regulated in malignant prostate cells in situ, we have proposed that ZIP1 is a tumor suppressor gene in prostate cancer. It is notable that zinc also exhibits tumor suppressor effects in other cancer cells (for review see Franklin and Costello 2007).

The Clinical Relevancy and Translational Application of Citrate Metabolism in Prostate Cancer

That altered citrate metabolism is a consistent hallmark characteristic that distinguishes prostate malignancy from normal and hyperplastic glands is well established by clinical and experimental studies. An important issue is how to employ this relationship for clinical application such as the diagnosis, prevention, and/or treatment of prostate cancer.

1. In situ MRS citrate detection of malignant prostate loci.

Presently, no highly reliable test for the detection of early stages of prostate cancer exists. The extensively and commonly employed PSA test is unreliable, especially in the elderly male population that generally exhibits BPH. Even the invasive biopsy examination often fails to identify a malignant prostate gland. A more accurate and preferably noninvasive or minimally-invasive diagnostic procedure is urgently needed. The unique citrate metabolism relationships of the prostate, coupled with recent developments and technological advancements in 1-H MRSI (magnetic resonance spectroscopy imaging) for the in situ determination of citrate

levels, provides an excellent noninvasive diagnostic/detection procedure that can achieve all these goals. The metabolic transformation of citrate-producing prostate epithelial cells to citrate-oxidizing prostate malignant cells provide the basis for the development of 1H-MRS imaging for the identification of malignant loci in the prostate gland. When combined with MRI a "metabolic map" of the prostate gland can be obtained, which reveals important metabolic relationships as shown in Fig. 5. MRSI reveals that the normal peripheral zone contains the high concentration of citrate as compared with lower levels of citrate in the central zone. Malignant loci are readily identified by the marked decrease and depletion of citrate. MRS clearly establishes that malignant loci virtually never retain the high citrate levels that characterize the normal peripheral zone. In contrast to the decrease in citrate, choline levels are increased in malignant loci, which likely reflect the increase in lipogenesis/cholesterogenesis associated with malignant cell activity. The consis-

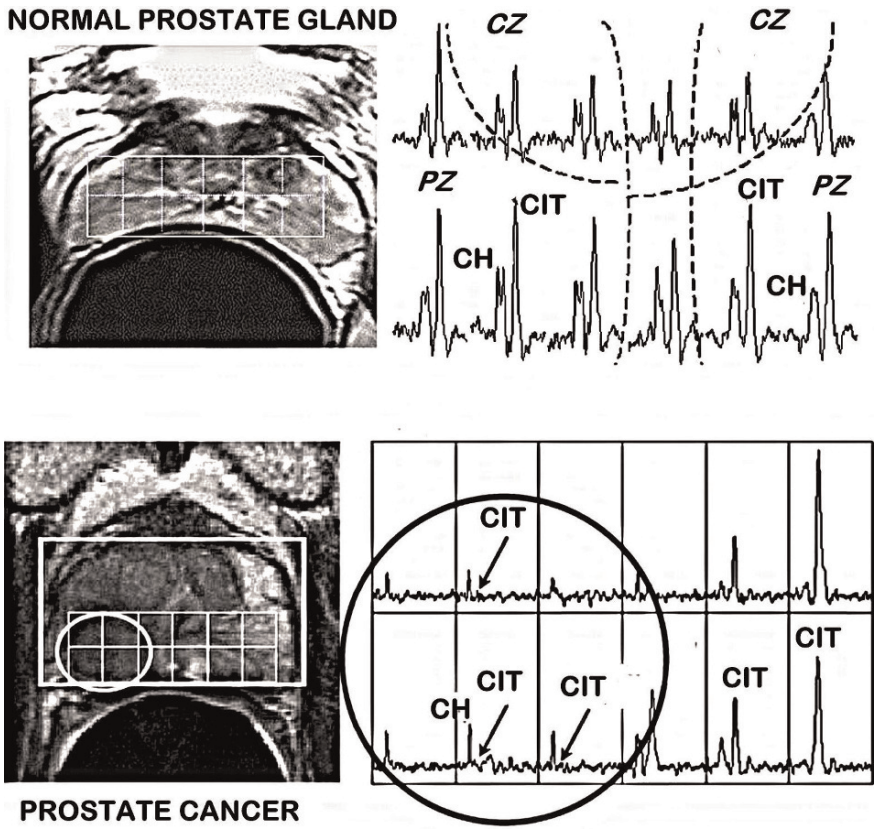


Fig. 5 In situ MRS imaging of citrate in the human prostate gland. In the normal gland, citrate is high in the peripheral zone (PZ) and low in the central zone (CZ). In prostate cancer, the malignant locus in the right peripheral zone is depleted of citrate compared with the high citrate in the left peripheral zone. CH: choline peak

tency and accuracy of the MRS imaging of citrate metabolism transformation now provides the most accurate method (~90–95% accuracy) for the identification, localization, and volume estimation of malignancy, and is even being developed for more accurate guided brachytherapy applications. Also evident is the fact that hyperplastic glands that occupy the central gland in the development of BPH are citrate-producing glands as differentiated from the normal central zone glands that are not high citrate-producing glands. For extensive reviews of MRS application in prostate cancer see Costello et al. (1999) and Kurhanewicz et al. (2002).

2. Evidence for a "premalignant" stage of malignancy: Identification of "at-risk" individuals.

The consistency of the decreased citrate associated with malignant prostate is striking. The citrate change in PCa is evident in citrate analysis of biopsy samples, in TURP samples, in prostatectomy samples, and in in situ measurements by MRSI. The citrate change is apparent even with large variations in the histological composition of the tissue samples being analyzed. The decreased citrate in PCa is detectable despite the admixture of normal, benign, and malignant epithelial cell populations within the sample being analyzed.

The MRS studies corroborate this premise in that the volume of the voxels analyzed often exceeds the actual malignant locus and include significant amounts of nonmalignant glandular tissue. Yet citrate is consistently markedly decreased when PCa has been identified. There exists virtually no malignant tissue that has retained the high citrate level characteristic of normal and BPH tissue. One must conclude that, by the time the onset of malignancy is established and identified by histopathological examination, a significant decrease in citrate levels has occurred in the region of the peripheral zone. A normally expect proportional gradation of citrate changes as the ratio of malignant vs. nonmalignant cells increases is not apparent. On the contrary, the marked decrease in citrate is manifested even when the malignant cell population occupies a small proportion of the volume of locus being examined. This is also evident in the report of Schiebler et al. (1993), which observed the low MRS citrate signal in a tissue sample that contained minimal malignant glands in a field dominated by normal glands. The most plausible explanation for this relationship is that a large population of glandular epithelial cells that do not produce citrate must exist by the time that overt malignancy has been established. The metabolic transformation must exist in a large population of neighboring cells that are not histopathologically identified as malignant cells, and we characterize these as "premalignant" cells (Fig. 3). The biochemical changes associated with malignancy precede the histopathological changes. This probably accounts, at least in part, for the absence of a proportional decrease in citrate as correlated with the Gleason grade.

The MRSI detection of prostate cancer includes a perceived false-positive result in which a peripheral zone decrease in citrate is not correlated with the histopathological identification of malignancy. This is likely due to an early, premalignant stage that identifies an "at-risk" individual. We submit that follow-up examinations of this individual over a period of one to two years are likely to reveal

the overt identification of malignancy. This metabolic mapping of the prostate gland will provide a record to compare the suspected locus as malignancy develops.

3. Prostatic fluid citrate as a biomarker for prostate cancer.

As shown in Table 1, normal prostatic fluid contains enormously high citrate levels that ranges from ~40–150 mM. Since the adenocarcinomatous glands are not citrate-producing glands, one would expect that the prostatic fluid from prostate cancer subjects would exhibit a marked decrease in citrate concentration. Interestingly, Huggins (1946) reported that prostate cancer prostatic fluid contained 20- to 40-fold less citrate than normal or BPH prostatic fluid. More recently, Kline et al. (2006) also reports a decrease in citrate content of prostate cancer prostatic fluid although the studies involved a small number of subjects. An extensive clinical study is necessary to establish the reliability and specificity of prostatic fluid citrate levels for the diagnosis of prostate cancer. This could provide a simple, rapid, noninvasive test for screening and detection of prostate cancer.

4. Targeting citrate metabolism for the treatment of prostate cancer.

That malignant prostate cells do not exist as citrate-producing cells provides the opportunity for new approaches to the treatment, and possibly prevention, of prostate cancer. Since the accumulation of cellular zinc is responsible for the prevention of citrate oxidation in the normal prostate cell, its restoration in malignant cells could arrest and/or abort the development of the malignant cells. However, the problem is to develop a zinc chaperone that will lead to zinc uptake and accumulation in malignant or premalignant prostate cells that have a downregulated expression of Zip1 transporter. Another approach is to develop an alternative inhibitor of citrate oxidation that might be specific for prostate cells. The establishment of conditions that result in reexpression of Zip1 in the malignant cells should result zinc accumulation and its tumor-suppressor effects. Such possibilities await much needed investigation.

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References

Baggetto, L. G. 1992. Deviant energetic metabolism of glycolytic cancer cells. Biochimie 74:959–974.

Costello, L. C., and Franklin, R. B. 1989. Prostate epithelial cells utilize glucose and aspartate as the carbon sources for net citrate production. Prostate 15:335–342.

Costello, L. C., and Franklin, R. B. 2001. The intermediary metabolism of the prostate: A key to understanding the pathogenesis and progression of prostate malignancy. Oncology 59: 269–282.

Costello, L. C., and Franklin, R. B. 2002. Testosterone and prolactin regulation of metabolic genes and citrate metabolism of prostate epithelial cells. Horm Metabol Res 34:417–424.

Costello, L. C., and Franklin, R. B. 2005. "Why do tumor cells glycolyze?": From glycolysis through citrate to lipogenesis. Mol Cell Biochem 280:1–8.
Costello, L. C., and Franklin, R. B. 2006. The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: Connecting the dots. Mol Cancer 5:17.
Costello, L. C., Franklin, R. B., and Stacey, R. 1976. Mitochondrial isocitrate dehydrogenase and isocitrate oxidation of rat ventral prostate. Enzyme 21:495–506.
Costello, L. C., Lao, L., and Franklin, R. B. 1993. Citrate modulation of high affinity aspartate transport in prostate epithelial cells. Cell Mol Biol 39:515–524.
Costello, L. C., Liu, Y., Franklin, R. B., and Kennedy, M. C. 1997. Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells. J Biol Chem 272:28875–28881.
Costello, L. C., Franklin, R. B., and Narayan, P. 1999. Citrate in the diagnosis of prostate cancer. Prostate 15:237–245.
Costello, L. C., Guan, Z., Kukoyi, B., Feng, P., and Franklin, R. B. 2004. Terminal oxidation and the effects of zinc in prostate and liver mitochondria. Mitochondrion 4:331–338.
Costello, L. C., Feng, P., and Franklin, R. B. 2005. Mitochondrial function, zinc, and intermediary metabolism relationships in normal prostate and prostate cancer. Mitochondrion 5:143–153.
Feng, P., Liang, J. Y., Li, T. L., Guan, Z. X., Zou, J., Franklin, R. B., and Costello, L. C. 2000. Zinc induces mitochondria apoptogenesis in prostate cells. Mol Urol 4:31–36.
Feng, P., Li, T. L., Guan, Z. X., Franklin, R. B., and Costello, L. C. 2002. Direct effect of zinc on mitochondrial apoptogenesis in prostate cells. Prostate 52:311–318
Franklin, R. B., and Costello, L. C. 2007. Zinc as an anti-tumor agent in prostate cancer and in other cancers. Arch Biochem Biophys 463:211–217.
Franklin, R. B., Lao, L., and Costello, L. C. 1990. Evidence for two aspartate transport systems in prostate epithelial cells. Prostate 16:137–146.
Franklin, R. B., Milon, B., Feng, P., and Costello, L. C. 2005. Zinc and zinc transporter in normal prostate function and the pathogenesis of prostate cancer. Frontiers Biosci 10:2230–2239.
Franklin, R. B., Zou, J., Yu, Z., and Costello, L. C. 2006. EAAC1 is expressed in rat and human prostate epithelial cells; functions as a high-affinity L-aspartate transporter; and is regulated by prolactin and testosterone. BMC Biochemistry 7:10.
Guan, Z., Kukoyi, B., Feng, P., Kennedy, M. C., Franklin, R. B., Costello, L. C. 2003. Kinetic identification of a mitochondrial zinc uptake transport process in prostate cells. J Inorganic Biochem 97:199–206.
Halliday, K. R., Fenoglio-Preiser, C., and Sillerud, L. O. 1988. Differentiation of human tumors from nonmalignant tissue by natural-abundance 13C NMR spectroscopy. Magn Reson Med 7:384–411.
Harkonen, P. L. 1981. Androgenic control of glycolysis, the pentose cycle and pyruvate dehydrogenase in the rat ventral prostate. J Steroid Biochem Mol Biol 14:1075–1084.
Huggins, C. 1947. The prostatic secretion. Harvey Lect 42:148–193.
Kline, E. E., Treat, E. G., Averna, T. A., Davis, M. S., Smith, A. Y., and Sillerud, L. O. 2006. Citrate concentrations in human seminal fluid and expressed prostatic fluid determined via 1H nuclear magnetic resonance spectroscopy outperform prostate specific antigen in prostate cancer detection. J Urol 176:2274–2279.
Krezel, A., Hao, Q., and Maret, W. 2007. The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling. Arch Biochem Biophys 463:188–200.
Kurhanewicz, J., Swanson, M. G., Nelson, S. J. and Vigneron, D. B. 2002. Combined magnetic resonance imaging and spectroscopic imaging approach to molecular imaging of prostate cancer. J Mag Reson Imag 16:451–463.
Lao, L., Franklin, R. B., and Costello, L. C. 1993. A high affinity L-aspartate transporter in prostate epithelial cells which is regulated by testosterone. Prostate 22:53–63.
McKeechan, W. L. 1982. Glycolysis, glutaminolysis and cell proliferation. Cell Biol Int Rep 6:635–650.

Moreadith, R. W., and Lehninger, A. L. 1984. The pathways of glutamate and glutamine oxidation by tumor cell mitochondria. Role of mitochondrial NAD(P)+-dependent malic enzyme. J Biol Chem 259:6215–6221.

Muntzing, J., Varkarakis, M. J., Saroff, J., and Murphy, G. P. 1975. Comparison and significance of respiration and glycolysis of prostatic tissue from various species. J Med Primatol 4:245–251.

Outten, C. E., and O'Halloran, T. V. 2001. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488–2492.

Parlo, R. A., and Coleman, P. S. 1984. Enhanced rate of citrate export from cholesterol-rich hepatoma mitochondria. J Biol Chem 259:997–10003.

Parlo, R. A., and Coleman, P. S. 1986. Continuous pyruvate carbon flux to newly synthesized cholesterol and the suppressed evolution of pyruvate-generated CO2 in tumors: Further evidence for a persistent truncated Krebs cycle in hepatomas. Biochim Biophys Acta 886:169–176.

Pedersen, P. L. 1978. Tumor mitochondria and the bioenergetics of cancer cells. Prog Exp Tumor Res 22:190–274.

Racker, E., and Spector, M. 1981. Warburg effect revisited: Merger of biochemistry and molecular biology. Science 213:303–307.

Schiebler, M. L., Schnall, M. D., Pollack, H. M., Lenkinski, R. E., Tomaszewski, J. E., Wein, A. J., Whittington, R., Rauschning, and W., Kressel, H. Y. 1993. Current role of MR imaging in the staging of adenocarcinoma of the prostate. Radiology 189:339–352.

Singh, K. K., Desouki, M. M., Franklin, R. B., and Costello, L. C. 2006. Mitochondrial Aconitase and Citrate Metabolism in Malignant and Nonmalignant Human Prostate Tissues. Mol Cancer 5:14.

Swinnen, J. V., Heemers, H., Heyns, W., and Verhoeven, G. 2002. Androgen regulation of lipogenesis. Adv Exp Med Biol 506:379–387.

Vallee, B. H., and Falchuk, K. H. 1993. Physiol Rev 73:79–118. The biochemical basis of zinc physiology.

Warburg, O., Wind, F., and Negelein, E. 1926. Uber den Stoffwechsel von Tumoren im Korper. Klin Woch 5:829–832.

Integration of Genetic, Proteomic, and Metabolic Approaches in Tumor Cell Metabolism

Leslie C. Costello and Renty B. Franklin

Abstract There now exists a resurgence of interest in and research into the role of altered cellular intermediary metabolism in the development and progression of cancer and other disease processes. The recent developments in molecular technology, molecular biology, molecular genetics, and proteomics provide new tools to investigate the involvement of the genetic and molecular factors that regulate the adaptation of the metabolism of malignant cells to meet the synthetic and bioenergetic requirements of the malignant process. Such didactic and technological capabilities that did not exist during the preceding generations of research in intermediary metabolism must be integrated with the biochemical/enzymological methods and principles of cellular enzyme activities and metabolic pathways. This requires that the contemporary and future investigators integrate the traditional cellular metabolic principles and methods with the molecular technological capabilities and didactic information to study the role of altered intermediary metabolism in malignancy. A guiding axiom is that "Genetic transformations and proteomic alterations will have little relevancy to tumor metabolism and other disease processes if the genetic/proteomic alterations are not manifested in altered and impaired cellular and metabolic function." In this chapter we discuss some important principles of cellular metabolism and the approaches employed to determine the metabolic adaptations involved in malignancy. We integrate the areas of cellular intermediary metabolism with molecular genetics, proteomics, and metabolomics to provide the basis for elucidation of the genetic/molecular/-metabolic factors in the development and progression of cancer.

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Introduction – In the Beginning

The hallmark studies of Otto Warburg and colleagues reported in 1926 (Warburg et al. 1926) sparked the era of tumor cell metabolism. From then until around 1980 studies of intermediary metabolism of normal and malignant cells were dominant areas of research and of graduate and postgraduate training in biomedical sciences. This era culminated in the outstanding discoveries of the operation of the Emden–Meyerhoff (glycolytic) pathway, the operation of the Krebs cycle, the pathway of terminal oxidation, the coupling of energy production through coupled oxidation–phosphorylation, and other associate pathways involved in the "thoroughfare" of cellular intermediary energy metabolism. The evolution of these discoveries resulted from the pioneering efforts of earlier generations of outstanding, dedicated, and tireless scientists to which the contemporary and future generations owe an enormous degree of respect, admiration, and appreciation.

The contemporary era of molecular technology, nanotechnology, microassays, computers, automation, and other such advances in analytical methods were non-existent and unavailable to the earlier generation and were not employed in those great discoveries. One could not identify, visualize, and quantify the existence of an enzyme in a cell or cell extract as we can now readily, rapidly, and easily achieve through Western blot analysis. One could not conduct multiple rapid microassays of cell reactions as can be achieved by instrumental analysis such as fluorescent microplate readers. Moreover, the research support services that facilitate the scientists of today were not available to the earlier generations. Data analyses and statistical analyses were performed manually or at best with the aid of a "sophisticated" calculator. Each scientist was also a trained statistician, and it would take hours and days to analyze the data from an experiment. Literature searches for methods and background publications were conducted at the library. This was achieved by the tedious and time-consuming process of looking through the index of volumes of issues of Chemical Abstracts, Biological Abstracts, Index Medicus, and other such index/abstract publications. The researcher had to manually write pages of abstracts and citations since copying machines and services were not available. Today, a computer-generated PubMed search from one's office or home will generate within minutes and with a hardcopy printout of the information that our predecessors could not obtain even within weeks of library "work". These are the conditions under which the great advances and discoveries were achieved.

Today's researchers have all the technological advances, instrumentation, and tools that make research in cell metabolism (and all other areas) much simpler, more rapid, more efficient, and more sensitive than their predecessors as well as new advances that permit investigations into issues that were not previously possible. Notwithstanding all of these technological and informational advances, the experimental approach and the requirements to establish the cellular activity of an enzyme and the operation of a metabolic pathway have not changed. There is no contemporary alternative to the essential biochemical/enzymological measurements that were employed by our predecessors. Better, simpler, more rapid, more

sensitive assay procedures and methods now exist, but the approach remains the same. The pioneers were expert in the areas of biochemistry, enzymology, enzyme reaction kinetics, metabolic principles, and the methods required to investigate and to establish cellular metabolic pathways and relationships.

This focus and expertise is woefully deficient in the didactic and technological training, capability, and understanding of a major segment of contemporary investigators and trainees. Beginning around 1980, the advent, development, and subsequence dominance of molecular genetics, proteomics, and molecular technology in clinical and experimental biomedical application were accompanied by the nearly complete submersion of interest and training in areas of intermediary metabolism and tumor cell metabolism. This is the issue that must be appreciated, addressed, and reconciled as we enter the rejuvenated era of the role of intermediary metabolism in the development and progression of malignant cells in all types of cancers. This provides a timely reason to revisit some of the important issues of tumor cell metabolism with a perspective of the contemporary associations of genomics/proteomics/metabolomics, coupled with molecular technology; none of which existed during the exciting glorious days of the outstanding biochemists and mitochondriacs of earlier times.

Relationships of Cellular Activity, Cellular Metabolism, and Malignancy – Some Important Axioms

The following are important generalizations that we consider to be axiomatic and applicable to all cells (Fig. 1).

1. The existing cellular intermediary metabolism of a cell provides the bioenergetic/synthetic/catabolic requirements essential for the manifestation of the cell's current activities (function, growth, and proliferation).
2. When the activity of a cell changes, its metabolism must also be adjusted consistent with any newly established bioenergetic/synthetic/catabolic requirements.
3. Malignant cells are derived from normal cells that have undergone a genetic transformation to a neoplastic cell phenotype that is endowed with malignant potential.
4. Manifestation of the malignant potential of the neoplastic cell necessitates alterations in its metabolism (i.e., a metabolic transformation) to provide the bioenergetic/synthetic requirements of malignancy.
5. In the absence of the metabolic transformation, the neoplastic cell will not progress to complete malignancy. Conversely, the metabolic transformation, in the absence of the genetic transformation to a neoplastic malignant cell, will not cause malignancy.
6. Genetic transformations and proteomic alterations will have little relevancy to tumor metabolism and other disease processes if the genetic/proteomic alterations are not manifested in altered and impaired cellular and metabolic function. (Note: This is a new axiom that arises from the contemporary development of molecular genetics and proteomics.)

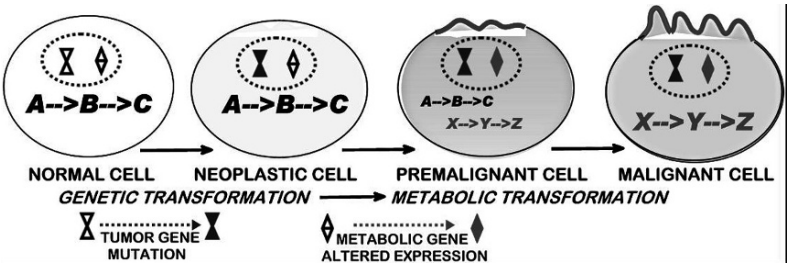

Fig. 1 The role of altered intermediary metabolism in the process of the development of malignancy. Malignancy begins with the genetic transformation of a normal cell to a neoplastic malignant phenotype. The neoplastic cell undergoes metabolic gene expression changes involved in the metabolic transformation from the normal cell metabolism of A→B→C to the malignant cell metabolism of X→Y→Z to fulfill the energetic and synthetic metabolic requirements of malignancy. The neoplastic cell can then fulfill its malignant potential as a malignant cell

Two Important Relationships that Establish the Metabolic Focus of Malignant Cells

In addressing and exploring the role of altered (in most cases) intermediary metabolism in the development and progression of malignant cells, one must be ever mindful of the importance of at least two (there are others) important relationships: (1) the metabolic requirements of malignant cells; and (2) the influence of the malignant cell–host tissue interrelationship on the metabolism of the malignant cells. This is especially relevant since most studies are conducted under in vitro conditions with tumor cell lines. The relationship of the experimental conditions and the characteristics of the experimental malignant cells to the realities of the "true" in situ malignant cells and their natural environment must be firmly considered in projecting the relevancy to cancer development and progression.

A Parasitic Existence Defines the Malignant Cell Metabolism

An understanding of the "purpose" of the existence of a cell at any point in time in its life provides information of the requirement for and role of its intermediary metabolism. The "parasitic" nature of malignant cells dictates that their metabolic activities are directed to support and manifest their existing purpose to grow and to proliferate to ensure their generational propagation. They do so by incorporating and utilizing the environment of their host tissues, which occurs at the expense and destruction of the host. The malignant cells exhibit two essential activities for their progression and propagation: (1) growth and proliferation; (2) invasion and motility. The latter are life-cycle activities in support of the former. The intermediary metabolism of the malignant cells must provide the bioenergetic, synthetic, and catabolic

requirements for these activities. The former dictates that the malignant cells become de novo lipogenic/cholesterogenic cells. In most cases the precursor sane cells that become neoplastic cells are not lipogenic/cholesterogenic cells. Therefore, the proliferating malignant cells must undergo a metabolic transformation directed at de novo lipogenesis/cholesterogenesis, which is required to meet the membraneogenesis requirement of proliferation. Coupled with this are other metabolic alterations to provide the protein, carbohydrate, and other components of net increase in cell number and cell mass. The bioenergetic requirements to support growth/proliferation as well as motility/invasion must be achieved. This is an example of the required metabolic adaptations in support of the manifestation of the malignant activities of the neoplastic cells. In the absence of these metabolic activities, the malignant cell would be in an arrested condition or would die.

The In Situ Environment of the Malignant Cell Dictates its Metabolism

Regardless of the metabolic requirements essential to their malignant activities, the malignant cells must achieve these metabolic requirements through their interaction with the host's tissue environment. The malignant cells are dependent upon the host tissue and circulation for their supply of oxygen and nutrients, and for the elimination/removal of the potentially toxic by-products of their metabolism. Especially for solid tumors, the malignant cells are subjected to a changing environment as they grow and progress. Most notable is the influence of the availability of oxygen and micronutrients derived from circulation. The former is of paramount importance in relation to the intermediary metabolism of the malignant cell. The initiation of the malignant cell activity is followed by growth and proliferation that results in an increasing mass of malignant cells. This subjects the population of malignant cells to different gradients of oxygen ranging from normoxia through hypoxia toward anoxia. One can visualize a solid ball of cells in which the outside layer of cells is in apposition to the air and each inner layer of cells progressively is more distant from the air. Thus, the intermediary metabolism of the malignant cells comprising the tumor mass cannot be expected to be uniform at any one time, and the intermediary metabolism of the malignant cells can be expected to change as the availability of oxygen changes. As the environment becomes more hypoxic leading to anoxia, the continued malignant proliferation and other activities will become compromised and eventually become arrested. This is due to inability of the major population of malignant cells to derive their metabolic bioenergetic and biosynthetic requirements. The lack of available oxygen and nutrients such as glucose from circulation prevents the malignancy from progressing. This is best illustrated by the requirements for lipogenesis/cholesterogenesis and even the accelerated glycolysis, neither of which can be sustained under such conditions. One must not forget that the "waste products" of the metabolism of the malignant cells also need to be eliminated, which also requires the availability of circulation.

In other words, a refurbished perfusate is an essential environmental condition for tumor progression. Indeed, the successful evolution of malignant cells has resulted from adaptive capabilities to confront and to overcome this adversity. For example, they upregulate hypoxia inducible factor and stimulate angiogenesis to create the circulation and environment that allows their further progression.

Therefore, the cycle of malignancy involves periods of growth and proliferation and periods of arrest to "refuel" the environment, all of which accommodate the metabolic requirements of the malignant cells. To optimize their parasitic existence, some malignant cells will vacate the primary site of their development and seek other host tissue sites to invade and to continue their parasitic existence. Thus, the capability and stages of metastasis are invoked. The vascularization and distant tissue site invasion provide different environmental conditions that likely affect the metabolism of the metastatic cells. Very little is known concerning the in situ metabolic relationships of these cells. The understanding of all of these relationships (and other relationships not described herein) dictate that any studies of tumor cell metabolism must be extrapolated and related to the realities of the in situ environment of the malignant cells.

Important Cellular Biochemical/Metabolic Relationships

In studying the cellular metabolism of tumor cells, and in fact any cells, one must have an understanding of some important relationships that govern enzyme activities and metabolic pathways. In any series of reactions that comprises a metabolic pathway, the activity rate of the pathway is governed by the slowest reaction within the pathway (the 'master reaction'). The following exemplifies a sequence of enzymes comprising a metabolic pathway leading to the following product:

Enzyme activities 1,2,4 are in excess, and enzyme 3 is rate limiting. The product of the pathway 'E' is low although enzyme 4 is in excess. Reaction 4 is low because the substrate D concentration is lower than the K_m for the reaction 4 enzyme. Therefore, the upregulation of enzyme 4 gene expression will have little, if any, effect on increasing the pathway for conversion of substrate 'A' to product 'E'. Moreover, the accumulation of intermediate C could induce a product inhibition of reaction 2, which then decreases product C, even if enzyme 2 is in excess. In such an example, the identification of altered expression of metabolic genes and of changes in the level of the corresponding enzymes does not establish changes in the cellular activity of the enzyme or the associated metabolic pathway. Conversely, the identification of altered enzyme activity of metabolic pathways does not identify the factors and cause of the altered metabolism. This is when the genetic/proteomic approach becomes a critical tool for understanding mechanisms of regulation of cellular metabolism.

The activity of an enzyme is dependent upon the concentration of the enzyme and by the existing conditions that establish the kinetics of the enzyme. The former is determined by the expression and biosynthesis of the enzyme in its active form. The latter is dependent upon numerous conditions such as substrate concentration, pH, cofactor requirements, allosteric effects of modulators, and interconversion of active and inactive forms. The most common way to determine the activity and kinetic properties of a specific enzyme is to employ cell extract preparations with additional purification followed be appropriate assay of the enzyme's activity. Generally, the conditions employed result in maximal activity of the enzyme. Although the potential activity of the enzyme can be obtained, such information does not likely represent the conditions that exist in situ in the cell. Therefore, other additional approaches must be employed to determine the cellular enzyme activity. Such approaches include substrate analysis and specific inhibitor studies. For example, one can treat cells with fluoroacetate to investigate the involvement of m-aconitase activity in the operation of the Krebs cycle. Then the cellular accumulation of citrate or the oxidation of citrate can be determined, which will provide information of the role of m-aconitase in this metabolic pathway.

Mitochondrial Enzyme/Metabolism Analyses

The conditions presented earlier are applicable to mitochondrial metabolism studies. However some additional conditions must also be considered. Often, one cannot employ cell studies to investigate specific mitochondrial metabolism relationships. Among a variety of reasons, the cellular uptake of substrates and their availability to the mitochondrial compartment imposes limitations and rate limiting conditions. To circumvent such problems, isolated mitochondrial preparations are generally employed. However, one must be careful in preparing isolated mitochondria. Many studies require that the mitochondria be intact and functional, but it is not easy to obtain such preparations. To insure the "integrity" of the preparation as intact tightly coupled mitochondria, one generally employs mitochondrial respiration (oxygen uptake) analysis. Such preparations should exhibit essentially no endogenous oxygen uptake. Upon addition of a permeable oxidizable substrate, some relatively low level of oxygen uptake will occur. However, the oxygen uptake should be increased several-fold upon the addition of ADP or by the addition of an uncoupling agent such as dinitrophenol. In the absence of such characteristics, the mitochondria are probable damaged, insufficiently washed, and/or contaminated with cellular debris.

Terminal oxidation and the electron transport system are major functional metabolic activities of mitochondria. The contemporary focus on the role of mitochondria such as mtDNA mutation, ROS production, and the involvement of the respiratory complexes 1–4 in prostate cancer has revitalized interest in such mitochondrial studies. The determination of electron transport activity is subject to the same enzyme/metabolic considerations as described above. When one isolates

mitochondria and determines the activity of the individual components of the electron transport chain, the information obtained generally relates to the potential maximal activity of that component. Such measurements, generally spectrophotometric analysis of the oxidation or reduction of appropriate ox/red indicators such as cytochrome c and ubiquinone, are readily obtained with sufficient mitochondrial preparations. To make such measurements with changes in the endogenous components imposes the condition of performing spectrophotometric analysis in highly turbid mitochondrial samples in which the turbidity and its changes during the spectral analysis must be corrected during the spectral analysis. To address this problem, Britton Chance had developed the dual wavelength procedure and instrument design for measurements of turbid samples. The measurement of the flux of electrons through the intact electron transport system is more appropriate to the functional relationship but is more difficult to establish. This involves the employment of spectral analysis by split beam spectrophotometry determination of the ox/red state of the various endogenous electron transport components upon the addition of an oxidizable substrate and/or selective inhibitors of specific sites in the electron transport chain. Virtually all of these methods and techniques are described throughout the various volumes of Methods In Enzymology edited by Colowick and Kaplan.

The Concept of Metabolic Genes

In molecular genetics the gene products are treated essentially as a homogeneous group of proteins, with no recognition of the special relationship of the proteins that exist as enzymes involved in cellular intermediary metabolism. This introduces serious consequences and misinterpretations of the role and existence of altered cellular intermediary metabolism based on such genetic and proteomic analysis. To address this issue we identify those genes that are involved in the expression of enzymes of intermediary metabolism as "metabolic" genes from those genes that are involved in the expression of other proteins such as structural/skeletal proteins and secretory/digestive enzymes. The latter group can be classified as "abundant" proteins that require increased expression level over a many-fold range. Enzymes of intermediary metabolism are not abundant proteins and exist in microabundant levels. In many instances the alterations in the level of regulatory enzymes of intermediary metabolism in the range of 1–2 folds will exhibit significant changes in the cellular enzyme activity. In fact, it makes no sense for such regulatory enzymes to be increased several-fold above the level required for their cellular maximal activity. One must recognize the important distinction between regulatory enzymes of intermediary metabolism and other enzymes/proteins.

The statistical parameters that are applied to microarrays and to RT-PCR for identification of significant changes in the expression of a gene are of serious consequence for the application to "metabolic genes". The statistical stringency that is applied to the analysis of typical microarray data is somewhat arbitrary and

designed to separate signal from noise. In order to reduce the rate at which significant differences in expression are falsely identified, the threshold for designating differences as being significant is often set higher (e.g., two-fold or greater) than might be expected for significant functional differences in metabolic enzyme activity. This introduces a potential for "false-negative" results that is more probable for metabolic genes than for other genes.

Cellular enzyme activities are dependent on their abundance and the cellular conditions that modify their kinetic activities. The determination of changes in metabolic gene expression and/or the respective protein level do not establish changes and or alterations in the cellular enzyme/transporter activity or the associated metabolic pathways. The prostate m-aconitase relationship provides one example of this issue. m-Aconitase enzyme level is relatively unchanged in normal prostate and malignant cells, but the activity of m-aconitase is markedly inhibited by zinc accumulation in the former and uninhibited in the latter in which zinc is not accumulated (Costello et al. 1997; Singh et al. 2006). This accounts for the important metabolic transformation of normal citrate-producing cells to citrate-oxidizing malignant cells. The accumulation or loss of zinc is the critical factor. Gene microarray or proteomic analysis would not detect this metabolic alteration, and this would lead to the misinterpretation that m-aconitase was not an important step in the development of prostate malignancy.

These genetic/molecular/metabolic relationships highlight the importance of recognizing a distinction between the regulation of the enzymes with changes in intermediary metabolism compared to other cellular activities.

The Application of Molecular Genetics and Proteomics to Tumor Cell Intermediary Metabolism

The preceding sections provide background information concerning some important relationships of metabolic implications in the study of tumor cell metabolism. There are numerous additional considerations of enzymolgy, enzyme kinetics, biochemistry and metabolism that have not been presented but must be recognized by those who become engaged in issues of cancer metabolism. This is especially relevant as we enter and develop the contemporary application of molecular technology/molecular genetics/proteomics to studies of intermediary metabolism.

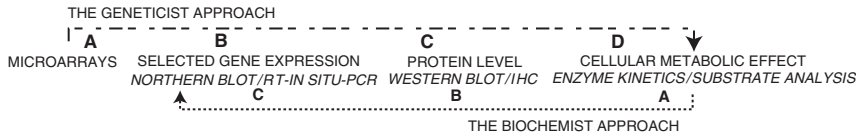

Fig. 2 The geneticist and biochemist approaches to establishing cellular enzyme activities and associated metabolic pathways in tumor cells

To address this issue we identify two approaches: the "*Biochemist Approach*" vs. the "*Geneticist Approach*" (Fig. 2).

The Biochemist Approach: In prior times, the study of cellular intermediary metabolism was conducted by those who were trained in the principles and methodology of biochemistry, enzymology, cellular physiology, metabolic pathways, and related areas. With this capability, these researchers identified the pathways of metabolism and the activities of the enzymes involved through the application of substrate analysis and enzyme kinetic studies; i.e. the "*Biochemist Approach*". To state that this was difficult, tedious, and time-consuming work is a gross understatement. As the molecular biology era evolved, that prior generation had to become trained in the developing areas of molecular genetics, proteomics, and molecular technology. For that group of investigators, the developing molecular approaches were added to their fundamental strength in biochemistry and metabolism. The view of that generation is that the cellular enzyme activities and operation of pathways are the critical events that need to be established. The role of gene expression and enzyme protein biosynthesis are viewed as critical tools to understanding the factors that are associated with alterations of cellular metabolism. Those investigators fully understand that molecular genetics and proteomics cannot identify the operation of cellular pathways of metabolism and/or specific enzyme activities; which can only be established by the traditional methods of substrate analysis and enzyme kinetics.

The *Biochemist approach* (Fig. 2) first seeks to identify the alteration in the cellular intermediary metabolism (step A) such as a change in the specific enzyme activity and/or the operation of a metabolic pathway. If an alteration in the cellular enzyme activity and/or associated metabolic pathway is not identified, its involvement in a metabolic transformation is unlikely. The need to proceed with genetic and proteomic studies (steps B and C), seemingly becomes unnecessary. However, pursuant genetic/proteomic studies might reveal altered expression and level of the enzyme. Then an important issue is revealed. "What are the cellular conditions that prevent the change in the activity of the altered enzyme level?" This would dictate the need for further investigation.

Alternatively, Step A might reveal a cellular alteration in the enzyme activity and associated metabolic pathway. Then, the issue becomes the identification of the mechanism of altered enzyme and metabolic activity. The application of the contemporary molecular tools of proteomics and gene expression are then applied, accompanied by biochemical examination of cellular conditions that can alter the activity of an enzyme. For example, a kinetic change in the enzyme V_{max} with no change in the substrate K_m value would suggest that the level of enzyme is altered. This could correlate with a corresponding change in the gene expression and/or protein level (steps C and B); and define a critical role of altered gene expression in the metabolic transformation. Conversely, the enzyme kinetic change might not be mimicked by genetic/proteomic changes. Then one must consider alternative reasons for the change in V_{max} as described earlier. There are other scenarios that exist. However, the "biochemist" approach is essentially devoid of potential false-positive and false-negative results relative to defining the involvement of specific

enzymes in metabolic transformations. The application of genetic/proteomic studies is essential for the elucidation of the mechanisms of altered enzyme activity and the regulation of metabolic transformations.

These relationships are also applicable to mutations in the mitochondrial genome. Mutated mtDNA is a common occurrence in malignant cells in situ. For example, mutations in the cytochrome c oxidase subunit (COX1 gene) have been widely reported in malignant prostate cells. However, important information concerning the effects of specific mutations on the mitochondrial cytochrome c oxidase activity and terminal oxidation often does not exist. Mutations that do not have any metabolic implications become irrelevant.

The Geneticist Approach: The preceding generations of biochemists are being replaced by a contemporary generation of molecular biologists who have been trained in and focused on the areas of molecular genetics, proteomics, and molecular technology. The principles and the technology of molecular genetics and proteomics are now being applied similarly and generally to proteins, among which enzymes of intermediary metabolism are included. These molecular events are then extrapolated to cellular metabolic events; i.e. the "*Geneticist Approach*". This now provides the opportunity that did not exist in prior generations to address the critical issues, *"What are the essential adaptive metabolic requirements of malignant cells, and how is the altered metabolism achieved?"*

However, this molecular biology/molecular technology dominance and emphasis (perhaps over-emphasis) have created problems and issues that did not exist in the past. These issues and problems must be recognized, addressed, and rectified. The weakness of this group of contemporary investigators is the absence of training in and understanding of the principles of biochemistry and enzymology, the factors that affect cellular enzyme activity, and the relationships of sequential enzyme activities in cellular metabolic pathways. Instead of integrating the didactic and technological training and experience of contemporary molecular biology with traditional understanding and application of the bichemistry/enzymology/metabolic principles, the latter has been largely supplanted and obliterated by the former.

When a phenotypic alteration (such as the transformation of the normal cell to a malignant cell), the *Geneticist approach* (Fig. 3) focuses initially on the identification of changes in gene expression by microarray analysis (step A) and/or specific gene expression analysis (step B). If a "significant" difference in a gene expression is revealed, studies proceed to the proteomic identification of corresponding changes in the relative level of the enzyme protein (step C). Very often a demonstrable alteration in the gene expression and the relative protein level become presumptive evidence of a corresponding change in the cellular enzyme activity and associated pathway of metabolism. This presumption leads to the geneticist approach ending at step C or by employing additional molecular genetic interventions such as knock-out and knock-in cells and animals. However, even when these experiments are carried out the effects of factors other than protein level may not always identify the physiological significance of the altered expression. None of these interventions should suffice for the elimination of the most critical step D.

If step D were to reveal an alteration in the cellular specific enzyme activity and/or the associated pathway due to the cellular kinetic conditions, the geneticist approach in the absence of step D would have elicited a "false-positive" interpretation. Conversely, if steps A and/or B and/or C reveal no significant change in the expression of a gene, the presumption is made that its associated enzyme and/or metabolic pathway is not involved in a metabolic transformation, and step D is eliminated. However, further study that involves step D could reveal cellular changes in the enzyme activity and associated pathway. Thus, a false-negative result would have been reported.

The genetic/proteomic approach to studies of cellular intermediary metabolism is not a replacement or substitute for the biochemical/enzymological approach. For this reason we have introduced the axiom, "*Genetic transformations and proteomic alterations will have little relevancy to tumor metabolism and other disease processes if the genetic/proteomic alterations are not manifested in altered and impaired cellular and metabolic function*". The contemporary literature contains numerous instances in which gene expression studies (e.g., RT-PCR) and protein abundance studies (e.g., Western blot analysis) have lead to conclusions that the changes in the expression and level of specific enzymes are evidence of corresponding changes in the cellular enzyme activity and associated pathway. Conversely, the absence of changes in expression has lead to conclusions that the enzyme-associated activity and pathway are not involved in altered metabolism in a tumor cell or a disease process. Notably absent from such reports are the essential cellular metabolic studies that are required to determine the relationship of genetic/proteomic observations to the cellular metabolic events. Such circumstances reveal the absence of an essential understanding of fundamental cellular metabolic relationships. The only circumstance in which a genetic/proteomic alteration can be directly related to a corresponding cellular enzyme effect is the complete down regulation of the gene with the absence of the enzyme; so that cellular enzyme activity cannot exist.

These issues in the marriage of molecular genetics/proteomics with cellular intermediary metabolism raise the important question, "What should be done to alleviate the problems?" Awareness of these issues and principles by the contemporary biomedical researchers will provide some immediate resolution in forthcoming studies and reports. The more permanent and meaningful resolution resides in the pre and postdoctoral training programs. Cellular biochemistry, intermediary metabolism, enzyme kinetics, and enzymology must be reinstituted into the didactic and seminar components of training of biomedical researchers; particularly in the molecular genetics training programs. Practical laboratory training and experience in the biochemical/metabolic methodologies along with the molecular technology methodologies would be extremely beneficial for those involved in studies of the regulation of cellular intermediary metabolism of tumor cells and other diseases. Such programs will provide the most capable generation of biomedical scientists to address and resolve the critical issues of altered intermediary metabolism in malignancy and in other areas of biology and medicine.

Integration of Intermediary Metabolism with Metabolomics in Cancer

As was pointed out, cells adapt their intermediary metabolism to meet the bioenergetic/synthetic requirements of the malignant process. The preceding presentation provides the integration of the biochemical and molecular tools employed in the identification of the enzymes and regulatory factors that comprise the existing cellular metabolic pathways. A new dimension referred to as "metabolomics" has evolved to provide a cellular or organism metabolic profile ("metabolome") that can be employed to identify or characterize genetic/metabolic alterations ("metabonomics") associated with cancer and other disease processes. For this discussion we will generically incorporate the term "metabolomics" and avoid the confusing, unsettled, subtle, and unnecessary distinctions in the meaning of these individual terms. In reality, "metabolomics" is not new. For example, the identification of the operation of the Krebs cycle as well as the events of the electron transport system evolved in part from "metabolomic" studies. These pathways constitute "metabolomes" as that term is currently defined and applied. The alterations in specific pathways of intermediary metabolism associated with the development of the malignant genotype/phenotype provide a changing metabolic profile that differentiates the malignant cell from the normal cell. An example is provided by the early study of Cooper and Farid (1963), who employed chromatographic analysis of metabolic intermediates in the identification of the loss of citrate in the development of prostate cancer cells, i.e., a change in citrate metabolism. This is confirmed by in situ magnetic resonance spectroscopy identification of prostate tissue metabolites that reveal a decrease in citrate and an increase in choline in malignant tissue.

The metabololmic approach requires the identification of a sequence of metabolites that constitute a metabolic pathway. For intermediates of metabolism, this generally involves the extraction of the intermediates from the cells/tissues, followed by the separation of the intermediates, followed by the quantitative determination of the concentrations of the intermediates. For some intermediates, direct identification can be achieved by techniques such as NMR and MRS. The contemporary metabolomic approach is essentially an application of the "biochemist approach" described earlier. Therefore we have returned to the earlier concepts of the important role of altered intermediary metabolism in the development and progression of malignant cells with the added advantages of contemporary developments in molecular biology and technology to elucidate the underlying factors and conditions responsible for the metabolic adaptations in cancer cells.

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References

Cooper, J.F., and Farid, I. 1963. The role of citric acid in the physiology of the prostate. A chromatographic study of citric acid cycle intermediates in benign and malignant prostatic tissue. J Surg Res 3:112–121.

Costello, L.C., Liu, Y., Franklin, R.B., and Kennedy, M.C. 1997. Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells. J Biol Chem 272:28875–28881.

Costello, L.C., Franklin, R.B., and Narayan, P. 1999. Citrate in the diagnosis of prostate cancer. Prostate 15:237–245.

Singh, K.K., Desouki, M.M., Franklin, R.B., and Costello, L.C. 2006. Mitochondrial Aconitase and Citrate Metabolism in Malignant and Nonmalignant Human Prostate Tissues. Mol Cancer 5:14.

Warburg, O., Wind, F., and Negelein, E. 1926. Uber den Stoffwechsel von Tumoren im Korper. Klin Woch 5:829–832.

Mitochondrial Respiration and Differentiation

Roberto Scatena, Patrizia Bottoni, and Bruno Giardina

Introduction

Cell differentiation is the process by which a progenitor cell progressively matures to assume its final morphology and function. Numerous signal transduction pathways contribute to this complex process, and many of these signal transduction pathways and their abnormalities are fundamental to the development and pathogenesis of cancer. Lack of differentiation (i.e., dedifferentiation, retro-differentiation, or anaplasia) is a morphological hallmark of malignant transformation and implies a reversion from a high level of differentiation to a lower level. The degree of dedifferentiation differs among cancers, and is often correlated to malignancy.

However, recent evidence has shown that cancers do not only represent "reverse differentiation" of mature, normal cell. Cancer may often arise from various maturation blocks in the so-called stem cells, which are pluripotent cells present in all specialized tissues that normally maintain the ability to differentiate into many cell types. However, it is clear that neither de-differentiation nor a maturation block can fully explain the intricate morphological, structural and functional characteristics of cancer cells. From a morphological point of view, the interesting features of neoplastic cells, that should be always considered when discussing on molecular biology of cancer, include (Vinay et al. 2004):

- ***Pleomorphism:*** Cells and nuclei characteristically display considerable variations in size and shape.
- ***Abnormal nuclear morphology:*** Nuclei are typically hyperchromatic and disproportionately large with respect to the cytoplasm; chromatin is often coarsely clumped and distributed along the nuclear membrane, and large nucleoli are usually present.

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- ***Mitoses:*** Undifferentiated tumors usually show large numbers of mitotic cells, reflecting greater proliferative activity. Unusual mitotic figures (tripolar, quadripolar, or multipolar spindles) are often present.
- ***Loss of polarity:*** Large masses of tumor cells grow in an anarchic, disorganized fashion.
- ***Giant cells:*** Large tumor cells with hyperchromatic nuclei may be present.
- ***Stromal alterations:*** The disproportion between cell growth and development of stromal support may cause large areas of ischemic necrosis in the tumor mass.

A fundamental question that remains to be fully clarified is the role of mitochondrial respiration in these marked cellular perturbations. It is not easy to define a precise role for mitochondrial respiration in cancer because the electron respiratory chain is closely interconnected with different functions and activities of the mitochondria and cell. Moreover, the role of mitochondria in cancer cells has been overlooked, essentially because of the well known metabolic shift to an "aerobic glycolysis" owing to mitochondria hypofunction and/or dysfunction. A detailed review of the Warburg effect and cancer cell metabolism is out of the scope of this discussion. However it must be stressed that cellular metabolism, and particularly oxidative mitochondrial metabolism, are totally subverted to the needs of cancer cells in which proliferation is the primary function.

Mitochondria play a role in various pathophysiological processes of cancer cells, in particular:

- ***Apoptosis:*** Mitochondria control an intrinsic caspase-mediated apoptosis pathway by releasing cytochrome c and other proapoptotic cofactors, including apoptosis inducing factor –AIF-, Smac/DIABLO, Omi/HtrA2 or endonuclease G from the intermembrane space into the cytoplasm. Membrane permeabilization is regulated by sensors/regulators of bioenergetic status, components of the permeability transition pore, proteins of the Bcl-2 family, and mitochondrial lipids (Cheng et al. 2006).
- ***Nuclear DNA mutations:*** Two classical mitochondrial enzymes, succinate dehydrogenase and fumarate hydratase, were shown to act as tumor suppressors. Mutations in these genes have been linked to some neoplasias with important vascular components. Accumulation of succinate and fumarate leaking to the cytosol inhibits a family of prolyl hydroxylase enzymes (PHDs). Depending on which PHD is inhibited, two different pathways that support tumor promotion may result. Cells may become resistant to certain apoptotic signals, or stabilization of hypoxia-inducible factor (HIF) may induce a pseudohypoxic response that contributes to the observed "aerobic glycolysis" of cancer cells (Brandon et al. 2006; King et al. 2006).
- ***Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS):*** Oxidative damage by free radicals seems to have a pathogenic role in different degenerative diseases and cancer (Droge 2002). The main sources of free radicals in cells are by-products of oxidative metabolism. Free radical damage may affect nuclear and mitochondrial DNA, proteins, and lipids. However,

recent data support a reevaluation of free radical toxicity, suggesting that physiological levels of these chemical species can function as intracellular signal transducers, and can even act as oncosuppressors (Takahashi et al. 2006). The hypothetical pathogenic role of free radicals in degenerative diseases and cancer may have been overestimated, and some conclusions can be influenced by laboratory artifacts (Herrnstadt et al. 2003; Salas et al. 2005). Nitric oxide has been shown to play a growing role in mitochondrial electron respiratory chain regulation and in the generation of cellular energy. New information about mitochondrial free radical production and their derivatives (above all in terms of H2O2 and NO) continue to highlight the potential pathogenic and therapeutic roles of these highly diffusible chemical species, independently by oxidative stress. In fact, evidence exists that these biological mediators can modulate mitogen activated protein kinases (MAPKs), fundamental components of phoshorylation and dephoshorylation signaling pathways involved in cell survival, proliferation, and differentiation (Moncada and Erusalimsky 2002; Cadenas 2004).

- ***Mitochondrial DNA (mtDNA) mutations:*** Similarly debated are mitDNA mutations (intragenic deletions, missense and chain-terminating point mutations, alterations of homopolymeric sequences, etc.) that some authors do not consider significant pathogenetic noxae in cancer but simple associated lesions or even effects of laboratory malpractice. This means that the role of mtDNA mutations and the consequent respiratory dysfunction in carcinogenesis ROS mediated pathways is still hardly debated (Brandon et al. 2006).
- ***Heat Shock Proteins (HSPs):*** HSPs are induced in many different cancer cells, and function to protect cells by blocking apoptosis during tumor progression. HSPs may also function to protect cells from other cancer characteristics such as genetic instability, ischemic stroma, Warburg effect, and invasion and metastatic potential, delineating a role in carcinogenesis also for mtHSPs, such as prohibitin, mortalin, HSP60/HSP10 complexes, and HSP-70 (Calderwood et al. 2006; Czarnecka et al. 2006).
- ***Glycolytic enzymes:*** As discussed earlier, cancer cells undergo aerobic glycolysis owing to expression of different glycolytic enzyme isoforms such as lactate dehydrogenase, phosphofructokinase, and hexokinase. Isoform II of hexokinase (HK II) is induced in various cancer cells and shares an intriguing interrelationship with mitochondrial oxidative metabolism. HK II typically binds to voltage-dependent anion channels (VDAC), located on the outer mitochondrial membrane, allowing direct access of enzyme to ATP generated by ATP synthase and transported across the inner-mitochondrial membrane by the adenine nucleotide translocator. In such a way, rapid synthesis of glucose 6 phosphate can maintain a highly glycolytic metabolic flux in malignant cells (Scatena et al. 2003; Gatenby and Gillies 2004; Mathupala et al. 2006; Moreno-Sanchez et al. 2007).

Mitochondrial Respiration and Cell Differentiation

Complex interrelationships between the different functions and activities of mitochondria make it difficult to identify a direct pathogenic mechanism linking respiration to differentiation/dedifferentiation in cancer cells. Protein expression profiles of normal vs. cancer cells show numerous qualitative and/or quantitative differences in several mitochondrial proteins. Dedifferentiated cancer cells show numerous differences in proteins involved in the electron respiratory chain. For example, a significant decrease in the β subunits of the F1 component of mitochondrial ATPase and an overexpression of ATPase inhibitor protein (IF1) have been described in hepatocellular carcinoma (Capuano et al. 1996; Modica-Napolitano and Singh 2004). Various studies confirmed a decrease of cytochrome oxidase in different neoplasias (Carreras et al. 2004; Krieg et al. 2004; Sanchez-Pino et al. 2007). Interestingly, other studies showed high transcript levels of ANT2 in several dedifferentiated renal carcinomas (Faure Vigny et al. 1996; Modica-Napolitano and Singh 2004). However the role of these mitochondrial alterations in the pathophysiology of cancer is still debated.

Later, we describe several recent studies investigating potentially significant links between mitochondrial respiration and cell differentiation in cancer.

Examination of glycolytic (hypoxic) and oxidative tumor phenotypes suggests that a reevaluation of energy metabolism and cellular respiration in cancer cells is necessary. New data confirm a more active role for mitochondrial physiology in cancer cell metabolism. Fantin et al. (2006) showed that attenuating LDH-A expression by short hairpin RNA (shRNA) in tumor cell lines induces stimulation of mitochondrial respiration and a decrease in the pathologically-elevated mitochondrial membrane potential. This observation implies that mitochondrial respiration in tumor cells may not be dysfunctional, and that LDH activity may regulate mitochondrial respiration. Moreover, LDH activity seems to influence the tumorigenicity of malignant cells. Alterations in mitochondrial membrane potential depend on the glycolytic phenotype of cancer cells, and the ratios of pyruvate/lactate and/or NADH/NAD + are fundamental signal transduction molecules for the mutually-regulated activities of LDH and mitochondrial electron respiratory chain. These data not only confirm a functional link between cancer cell differentiation and mitochondrial respiration, but underline the potential role of these interrelationships in clinical applications such as diagnosis, prognosis, and therapy of cancer.

Other studies have shown a link between cell respiration and cancer cell differentiation/dedifferentiation. An interesting example of this link is the effect of estrogens on cancer cells. The role of estrogen in breast cancer has been extensively studied, even if the molecular details of tumor promotion are still debated. Recently, it has been shown that estrogens can induce proliferation in an estrogen receptor-deficient cell line (HEK-293). Other findings have implicated mitochondria in estrogen-mediated alteration of cell proliferation. The mitochondrial peripheral benzodiazepine receptor plays a role in the dysregulation of human breast cancer cell growth, and estrogens influence the expression of different cell cycle genes in human breast cancer via mitochondria (Felty and Roy 2005).

Estrogen directly influences mitochondrial function by "nongenomic effects," but it is not clear what these precise mechanisms are at the mitochondrial level. Some authors have identified estrogen receptor subtypes α and β within mitochondria, suggesting a role for estrogen in mitochondrial transcription regulation. Mitochondrial transcription has been shown to be enhanced by estrogen treatment, resulting in an increase of subunits I, II, III, and IV of cytochrome oxidase and induction of NADPH dehydrogenase subunit I. Interestingly, estrogen treatment altered overall mitochondrial respiration by acting on all components of the electron respiratory chain (NADH dehydrogenase complex I, succinate dehydrogenase complex II, cytochrome bc1 reductase complex III, and cytochrome c oxidase complex IV) and by inhibiting mitochondrial ATP synthase complex V as well. In ATP synthase complex V, distinct binding sites for various estrogens are present (McEnery et al. 1989; Zheng and Ramirez 1999). Estrogens are highly lipophilic, and may interact with mitochondria via its hydrophobic membrane protein components. Estrogens can modulate ROS production at the level of electron respiratory chain complexes I and III, and production of mitochondrial ROS may alter various signaling pathways such as PKC, p53, extracellular regulated kinases, NF-κB, and the c-fos/c-jun heterodimer AP-1, eventually resulting in growth stimulation of estrogen-sensitive cells (Felty and Roy 2005). The precise molecular targets by which estrogen-induced oxidative stress causes oncogenesis requires further clarification.

Peroxisome Proliferator-Activated Receptors (PPARs) are a class of nuclear receptors involved in lipid and glucidic metabolism, immune regulation, and cell differentiation. Owing to the wide spectrum of biological activities, intensive pharmacological research has produced various synthetic PPAR ligands and activators with different affinities for various receptor subtypes (Michalik et al. 2004). Experimental and clinical studies have shown that the pharmacotoxicological profile of these drugs cannot be simply ascribed to activation of their specific receptors (Youssef and Badr 1998; Kersten et al. 2000; Palakurthi et al. 2001). Marked side effects of these drugs, such as rhabdomyolysis, acute liver failure, heart failure, and renal dysfunction may provide further evidence of additional molecular pathways involved in PPAR ligand action (Gale 2001; Isley 2003; Nesto et al. 2004). Interestingly, PPAR ligands induced cancer cell differentiation, originally in cancers that typically expressed PPARs (for example, thiazolidinediones differentiated liposarcoma, which present subtype γ PPAR) (Michalik, Desvergne and Wahli 2004). However, recent evidences indicate that these molecules can also induce cell differentiation in cancers with low or absent PPAR expression. Moreover other studies confirmed that the antitumor activities of such drugs do not depend on PPAR activation (Scatena et al. 1999; Palakurthi et al. 2001; Clay et al. 2002; Baek et al. 2003; Michalik et al. 2004).

Our experience with PPAR synthetic ligands leads us to hypothesize that these drugs induce cancer cell differentiation by a mechanism related to mitochondrial electron respiratory chain dysfunction. Data show that thiazolidinediones and fibric acid derivatives may induce differentiation in different tumor cell lines. Specifically, our studies have shown the following series of findings:

1. Bezafibrate inhibited cell proliferation in human leukemia cell lines HL-60, U-937, and K-562 in a dose-dependent manner. In HL-60, cell growth inhibition was associated with an increased number of cells in G0/G1 phase and a significant decrease in G2/M phase. Analysis of cell differentiation markers (CD) showed a dose-dependent increase in expression of CD11b and CD14 on HL-60 and of CD14 on U-937 cells. Functional assays confirmed that the phenotype was a more mature type. Both HL-60 and U-937 cells showed a dose-dependent restoration of the respiratory burst stimulated by PMA and zymosan. K-562 erythroleukemia cells showed a dose-dependent increase of hemoglobin synthesis. A similar cellular differentiation was observed upon treatment with two additional fibrate derivatives (clofibric acid and gemfibrozil). We also found that fibrate-induced differentiation was partially inhibited by antioxidants such as acetylcysteine (NAC), and that cells treated with fibrates showed mitochondrial damage (Scatena et al. 2003).
2. Some connections exist between cancer and mitochondria. There is a precise molecular mechanism at the basis of cancer-associated mitochondrial dysfunction and it broadly affects cellular metabolism, including oxidative metabolism in particular. Peculiar peroxisome proliferator activity has potential links with fibrate-induced mitochondrial damage and fibrate carcinogenic activity in rodents (Issemann and Green 1990). The compensatory machinery (i.e., HSP induction) thought to maintain cellular homeostasis has been implicated in promotion of cancer cell differentiation, and may be involved in peripheral modulation of oncogene and oncosuppressor expression.
3. Mitochondrial dysfunction and its consequences on cellular metabolism and differentiation need a careful evaluation. Interestingly, bezafibrate specifically inhibited the activity of NADH cytochrome c reductase in a dose-dependent manner in both HL-60 and in TE-671 (rhabdomyosarcoma) cells, while other mitochondrial respiratory chain enzyme activities did not change. The impairment of NADH oxidation induced a cellular metabolic shift towards anaerobic glycolysis, and β-oxidation, as shown by the dose-dependent increase of metabolites (lactate, alanine, glycolytic and not glycolytic acetate). The greater involvement of glycolysis was confirmed by significant phosphofructokinase induction and increase of glucose utilization in drug-treated cultures. Differentiation assays showed a dose-dependent recovery of the oxidative burst and inhibition of cell proliferation in HL-60 cells treated with bezafibrate. Fundamental observation was the correlation between mitochondrial dysfunction, metabolic shift and differentiation activity in cells treated with scalar doses of bezafibrate. Furthermore, quantitative comparison, on a molar ratio basis, between different PPAR ligands (bezafibrate, clofibric acid, gemfibrozil and ciglitazone) in terms of inhibition of NADH cytochrome c reductase activity, differentiation potency and antiproliferative index confirmed the presence of a strict correlation between these parameters (Scatena et al. 2003). These results suggest:

i. Inhibition of mitochondrial NADH dehydrogenase contributes to the pharmacological and toxicological profiles of both fibrate derivatives (strong hypolipidemic/weak hypoglycemic effect) and thiazolidinediones (hypoglycemic/insulin sensitizer effect). In terms of mitochondrial oncology, this may suggest a possible molecular mechanism at the basis of peroxisome proliferators activity and carcinogenic properties of fibrates, typically observed in rodents.
ii. A correlation between cellular respiration dysfunction, stimulation of anaerobic glycolysis, and induction of cancer cell differentiation strongly highlight the role of mitochondria and oxidative metabolism in the pathophysiology of cancer. Moreover, the intriguing data about the induction of a differentiation state associated to a shift towards an anaerobic glycolysis (paradoxical Warburg effect) confirm the need to reconsider cancer cell metabolism in general and the so-called Warburg effect in particular.
iii. Results linking mitochondrial respiratory chain dysfunction with induced metabolic adaption to a more differentiated phenotype suggests that the role of some oncogenes/oncosuppressors in cancer pathogenesis should be reevaluated.
iv. The existence of a transduction pathway master signal at the basis of a complex cellular reorganization program, in which ROS, NO, RNS form an important branch of the program and the NADH/NAD + ratio also plays a significant role.

4. The correlations among NADH dehydrogenase dysfunction, the paradoxical Warburg effect, and cancer cell differentiation suggest that further study is required. Considering the embryonal origin, the well-known hepatocarcinogenity of fibrates in rodents, and the potential metabolic implications, an interesting experimental model is the human hepatocarcinoma cell line Hep-G2. Fibrates and thiazolidinediones caused a dose-dependent mitochondrial dysfunction due to partial impairment of NADH dehydrogenase with compensatory stimulation of glycolysis, and above all a differentiation of hepatocarcinoma cell line characterized by: inhibition of cell proliferation, restoration of proteins secreted by the liver (albumin, transferrin, and cholinesterase), reduction of α-fetoprotein. Our proteomics data revealed an intriguing induction of HSP70 and an opportune modulation of protein expression pattern with oncogene and antioxidant functions (stathmin 1, DJ-1 protein, peroxiredoxin 2, nucleoside diphosphate kinase A). Notably, these experiments also showed a correlation between drug-induced mitochondrial complex I dysfunction and the capacity to stimulate glycolysis and cellular differentiation. For the different drugs tested, the ability to stimulate a cellular differentiation, induced by mitochondrial derangement, can be described as clofibric acid $\leq$ bezafibrate $<$ gemfibrozil $<<$ ciglitazone. Interestingly, the increase in glucose utilization by the Hep-G2 cell line was different from the other human cell lines studied, and did not correlate with mitochondrial dysfunction. These data might be explained

by drug-induced differentiated phenotype reactivated gluconeogenesis in the presence of large quantities of substrates such as lactate, acetate, and alanine (Scatena et al. 2004; Scatena et al. 2007). These data further confirm the link between cell differentiation and mitochondrial respiration, indicating that during cancer cell proliferation, mitochondria provide energetic and biosynthetic functions that are normally found only during embryonic or fetal stages. In particular, during tumor progression, a metabolic condition in which both glycolysis and the Krebs cycle are strongly stimulated to mainly generate a series of metabolic intermediates required for amino, fatty and nucleic acid synthesis. Moreover the mitochondrial electron respiratory chain preferentially utilizes alternative pathways such as alpha glycerophosphate and fatty acyl CoA as source of reducing equivalents. These results suggest an expansion of the role of NADH and the NADH/NAD + ratio in modulating a signal transduction pathway that controls differentiation/dedifferentiation status. They also suggest intercommunication between complex I and complex II to adapt cell metabolism to meet different physiopathological conditions.

Conclusion

Here we have provided a brief review of the mitochondrial respiration and cell differentiation literature together with an in-depth discussion of our own findings. The paucity of studies on the role of mitochondria in oncology is most likely due to the difficulty in separating the interconnected mitochondrial functions (for example, it is hard and even misleading to separately analyze the mechanism at the basis of mitochondrial apoptosis without considering the activity of permeability transition pore and, thereby, of mitochondrial membrane potential, and further, of oxidative phoshorylation and so on). In our opinion, many mitochondrial pathophysiological studies show conflicting results because the employed experimental models have focused only on a specific activity and neglected compensatory and redundant mechanisms.

Our experiences confirm the results of several other studies in suggesting a more active role of mitochondria in cancer and requiring a revision of the so-called "Warburg effect." Importantly, evidence of a strict correlation among oxidative phosphorylation, glycolysis, and cancer cell phenotype does not mean that dysfunctions in the mitochondrial oxidative metabolism induce cancer, but rather underlines that cancer cell adopts, by a strong selective program, a particular metabolic profile that tend to utilize every available metabolic pathway to fulfil its only purpose, i.e., proliferation.

Understanding the basis of molecular mechanisms that function in the interrelationships between mitochondrial respiration and cancer cell differentiation will have important clinical implications for cancer diagnosis, prognosis, and therapy. Moreover, the elucidation of molecular mechanisms involved in cancer cell differ-

entiation is fundamental for all potential clinical applications related to the physiopathology of stem cells.

References

Baek, S.J., Kim, J.S., Nixon, J.B., Di Augustine, R.P., and Eling, T.E. 2003. Troglitazone, a peroxisome proliferator-activated receptor (PPAR γ) ligand, selectively induces the early growth response-1 gene independently of PPAR γ. J Biol Chem 278: 5845–5853.

Brandon, M., Baldi, P., and Fallace, D.C. 2006. Mitochondrial mutations in cancer. Oncogene 25: 4647–4662.

Cadenas, E. 2004. Mitochondrial free radical production and cell signaling. Mol Aspects Med 25: 17–26.

Calderwood, S.K., Khaleque, M.A., Sawyer, D.B., and Ciocca, D.R. 2006. Heat shock proteins in cancer: Chaperones of tumorigenesis. Trends Biochem Sci 31: 164–172.

Capuano, F., Varone, D., D'Eri, N., Russo, E., Tommasi, S., Montemurro, S., Prete, F., and Papa, S. 1996. Oxidative phosphorylation and F0F1 ATP synthase activity of human hepatocellular carcinoma. Biochem Mol Biol Int 38: 1013–1022.

Carreras, M.C., Franco, M.C., Peralta, J.G., and Poderoso, J.J. 2004. Nitric oxide, complex I, and the modulation of mitochondrial reactive species in biology and disease. Mol Aspects Med 25: 125–139.

Cheng, W.C., Berman, S.B., Ivanovska, I., Jonas, E.A., Lee, S.J., Chen, Y., Kaczmarek, L.K., Pineda, F., and Hardwick, J.M. 2006. Mitochondrial factors with dual roles in death and survival. Oncogene 25: 4697–4705.

Clay, C.E., Monjazeb, A., Thorburn, J., Chilton, F.H., and High, K.P. 2002. 15-Deoxy-D12,14-prostaglandin J2-induced apoptosis does not require PPAR-γ in breast cancer cells. J Lip Res 43: 1818–1828.

Czarnecka, A.M., Campanella, C., Zummo, G., and Cappello, F. 2006. Mitochondrial chaperones in cancer: From molecular biology to clinical diagnostics. Cancer Biol Ther 5: 714–720.

Droge, W. 2002. Free radicals in the physiological control of cell function. Physiol Rev 82: 47–95.

Fantin, V.R., St-Pierre, J., and Leder, P. 2006. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 9: 425–434.

Faure Vigny, H., Heddi, A., Giraud, S., Chautard, D., and Stepien, G. 1996. Expression of oxidative phosphorylation genes in renal tumors and tumoral cell lines. Mol Carcinog 16: 165–172.

Felty, Q., and Roy, D. 2005. Estrogen, mitochondria, and growth of cancer and non-cancer cells. J Carcinog 4: 1–18.

Gale, E.A. 2001. Lessons from the glitazones: A story of drug development. Lancet 357: 1870–1875.

Gatenby, R.A., and Gillies, R.J. 2004. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 4: 891–899.

Herrnstadt, C., Preston, G., and Howell, N. 2003. Errors, phantoms and otherwise, in human mtDNA sequences. Am J Hum Genet 72: 1585–1586.

Isley, W.L. 2003. Hepatotoxicity of thiazolidinediones. Expert Opin Drug Saf 2: 581–586.

Issemann, I., and Green, S. 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347: 645–650.

Kersten, S., Desvergne, B., and Wahli, W. 2000. Roles of PPARs in health disease. Nature 405: 421–424.

King, A., Selak, M.A., and Gottlieb, E. 2006. Succinate dehydrogenase and fumarate hydratase: Linking mitochondrial dysfunction and cancer. Oncogene 25: 4675–4682.

Krieg, R.C., Knuechel, R., Schiffmann, E., Liotta, L.A., Petricoin, E.F. III, and Herrmann, P.C. 2004. Mitochondrial proteome: Cancer-altered metabolism associated with cytochrome c oxidase subunit level variation. Proteomics 4: 2789–2795.

Mathupala, S.P., Ko, Y.H., and Pedersen, P.L. 2006. Hexokinase II: Cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. Oncogene 25: 4777–4786.

McEnery, M.W., Hullihen, J., and Pedersen, P.L. 1989. F0 "proton channel" of rat liver mitochondria. Rapid purification of a functional complex and a study of its interaction with the unique probe diethylstilbestrol. J Biol Chem 264: 12029–12036.

Michalik, L., Desvergne, B., and Wahli, W. 2004. Peroxisome proliferator-activated receptor and cancers: complex stories. Nat Rev Cancer 4: 61–70.

Modica-Napolitano, J.S., and Singh, K.K. 2004. Mitochondrial dysfunction in cancer. Mitochondrion 4: 755–762.

Moncada, S., and Erusalimsky, J.D. 2002. Does nitric oxide modulate mitochondrial energy generation and apoptosis? Nat Rev Mol Cell Biol 3: 214–220.

Moreno-Sanchez, R., Rodriguez-Enriquez, S., Marin-Hernandez, A., and Saavedra, E. 2007. Energy metabolism in tumor cells. FEBS J 274: 1393–1418.

Nesto, R.W., Bell, D., Bonow, R.O., Fonseca, V., Grundy, S.M., Horton, E.S., Le Winter, M., Porte, D., Semenkovich, C.F., Smith, S., Young, L.H., and Kahn, R., 2004. Thiazolidinedione use, fluid retention, and congestive heart failure: A consensus statement from the American Heart Association and American Diabetes Association. Diabetes Care 27: 256–263.

Palakurthi, S.S., Aktas, H., Grubissich, L.M., Mortensen, R.M., and Halperin, J.A. 2001. Anticancer effects of thiazolidinediones are independent of peroxisome proliferator-activated receptor g and mediated by inhibition of translation initiation. Cancer Res 61: 6213–6218.

Salas, A., Yao, Y.G., Macaulay, V., Vega, A., Carracedo, A., and Bandelt, H.J. 2005. A critical reassessment of the role of mitochondria in tumorigenesis. PLoS Med 2: 296.

Sanchez-Pino, M.J., Moreno, P., and Navarro, A. 2007. Mitochondrial dysfunction in human colorectal cancer progression. Front Biosci 12: 1190–1199.

Scatena, R., Nocca, G., Sole, P.D., Rumi, C., Puggioni, P., Remiddi, F., Bottoni, P., Ficarra, S., and Giardina, B. 1999. Bezafibrate as differentiating factor of human myeloid leukemia cells. Cell Death Differ 6: 781–787.

Scatena, R., Bottoni, P., Vincenzoni, F., Messana, I., Martorana, G.E., Nocca, G., De Sole, P., Maggiano, N., Castagnola, M., and Giardina, B. 2003. Bezafibrate induces a mitochondrial derangement in human cell lines: A PPAR-independent mechanism for a peroxisome proliferator. Chem Res Toxicol 16: 1440–1447.

Scatena, R., Bottoni, P., Martorana, G.E., Ferrari, F., De Sole, P., Rossi, C., and Giardina, B. 2004. Mitochondrial respiratory chain dysfunction, a non receptor-mediated effect of syntetic PPAR-ligands. Biochemical and pharmacological implications. Biochem Biophys Res Comm 319: 967–973.

Scatena, R., Bottoni, P., Martorana, G.E., Vincenzoni, F., Botta, G., Pastore, P., and Giardina, B. 2007. Mitochondria, ciglitazone and liver: A neglected interaction in biochemical pharmacology. Eur J Pharmacol 567: 50–58.

Takahashi, A., Ohtani, N., Yamakoshi, K., Iida, S., Tahara, H., Nakayama, K., Nakayama, K.I., Ide, T., Saya, H., and Hara, E. 2006. Mitogenic signalling and the p16INK4a-Rb pathway cooperate to enforce irreversible cellular senescence. Nat Cell Biol 8: 1291–1297.

Vinay, K., Abul, K., and Nelson, F. 2004. Neoplasia. In: Robbins and Cotran Pathologic Basis of Disease 7th Edition, pp. 270–343. Elsevier Saunders: Philadelphia, PA.

Youssef, J., and Badr, M. 1998. Extraperoxisomal targets of peroxisome proliferators: Mitochondrial, microsomal, and cytosolic effects. Implications for health and disease. Crit Rev Toxicol 28: 1–33.

Zheng, J., and Ramirez, V.D. 1999. Purification and identification of an estrogen binding protein from rat brain: Oligomycin sensitivityconferring protein (OSCP), a subunit of mitochondrial F0F1-ATP synthase/ATPase. J Steroid Biochem Mol Biol 68: 65–75.

Integration of Energy Metabolism and Control of Apoptosis in Tumor Cells

John G. Pastorino and Jan B. Hoek

Introduction

Poorly differentiated and rapidly growing tumor cells tend to have higher glycolytic rates than noncancerous cells and these high rates of glycolysis occur even under conditions where O_2 is plentiful, resulting in high rates of lactate formation (Weinhouse 1972; Pedersen et al. 2002; Gatenby and Gillies 2004; Moreno-Sanchez et al. 2007; Kim and Dang 2006). These observations indicate that the feedback control mechanisms that normally regulate the balance between glycolysis and mitochondrial oxidation under aerobic conditions are less effective in tumor cells. Although this unusual feature of energy metabolism in tumor cells (commonly referred to as the 'Warburg effect') was first described more than 80 years ago (Warburg 1930), the recent resurgence of interest in the integration of metabolism with apoptosis and growth control has brought a renewed focus on the underlying mechanisms. Recent studies have provided evidence of a plethora of unexpected changes in energy metabolism that are associated with the cancer phenotype and drugs that target these pathways have emerged as potential novel cancer therapies. Importantly, these studies also highlight pathways by which normal cells integrate metabolism with the control of growth and differentiation and how they are equipped to handle the balance of survival and cell death or senescence in response to stress or during aging. Thus, these findings have broad implications not only for cancer and its treatment, but also for many other physiological or disease phenotypes, including diabetes, obesity, alcoholism, and aging.

What are the mechanisms by which the glycolytic advantage is maintained in tumor cells? A large number of studies have demonstrated a marked overexpression of specific isoforms of glycolytic enzymes in tumor cells. For instance, evidence has been reported for increased expression of the GLUT1 glucose transporter,

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hexokinase II, phosphoglucomutase (PGM), the M2-isoform of pyruvate kinase (M2-PK), lactate dehydrogenase-A, and certain isoforms of the lactate transporter (see later text). However, the control of glycolytic flux in normal cells is usually distributed across multiple steps and depends on the metabolic conditions of the cell. The metabolic conditions under which tumor cells operate can be subject to enormous changes, depending on access to oxygen and glucose (or other substrates) and their ability to dispose of metabolites such as lactate and to maintain pH balance. Rapidly growing tumors often have areas of limited blood supply that are hypoxic or have limited access to glucose or other substrates, whereas nearby areas are well supplied. Therefore, the ability to survive under such variable and stressful conditions is essential for tumor cells. How changes in expression levels of individual glycolytic proteins affect the rate of glycolysis in the tumor cells and the steady state levels of glycolytic intermediates depends critically on multiple factors other than the enzyme activity itself. These include not only the substrate and oxygen supply conditions and the relative activities of other enzymes of the main pathway, but also on the regulatory proteins and signaling factors that regulate glycolytic flux. Therefore, despite the multitude of reports on changes in critical enzymes in glycolysis and mitochondrial electron transport, the implications of these changes for energy homeostasis in the tumor cell are not readily predicted. Classical metabolic control analysis (Kacser and Burns 1973; Fell 1997) demonstrates that there is an uneven distribution of the control of the flux exerted by individual enzymatic steps in complex metabolic pathways, such as glycolysis and oxidative phosphorylation (The flux control of a particular enzyme is characterized by the control coefficient, defined as the change in flux through the pathway resulting from a small change in activity of that enzyme (Kacser and Burns 1973; Fell 1997, 1998)). How changes in the activities of specific enzymes of glycolysis and oxidative phosphorylation affect the control distribution of glycolytic flux and related pathways of cellular energy metabolism in tumor cells has not been carefully studied.

Overexpression of glycolytic enzymes may have other consequences than merely contributing to glycolysis and cellular energy supply and these may further integrate metabolism with the control of apoptosis. For instance, studies in our laboratory demonstrated that hexokinase II binding to mitochondria through the outer membrane protein VDAC protects them against the onslaught of pro-apoptotic factors (Pastorino et al. 2002).

More surprisingly, other recent studies demonstrated that a loss of mitochondrial energy metabolism may also contribute to the tumor phenotype. Defects in electron transport, in TCA cycle activity or in the regulation of the pyruvate dehydrogenase complex, have been associated with certain types of tumor. A functional interconnection between the loss of mitochondrial activity and tumorigenesis is suggested by studies showing that targeting the mitochondrial defects may be beneficial. Although it remains largely unclear by what mechanisms a suppression of mitochondrial energy metabolism would help enhance glycolysis, or how effects on cellular energy metabolism interrelate with changes in apoptotic control, recent studies have identified multiple crossovers where signaling pathways that control apoptosis also influence energy metabolism.

The armament tumor cells require to survive stress conditions and to maintain a high rate of proliferation often involves defenses against apoptotic processes that normally dispose of cells exposed to undue stress or which are energetically over-challenged or damaged. The control of apoptosis is closely interlinked with cellular energy metabolism, since mitochondrial (dys)function is a critical element in both. Not surprisingly, the cellular signaling processes that control the balance of survival and apoptosis have recently also been found to affect energy metabolism. For instance, the Akt signaling pathway, a critical regulator of cellular survival and growth control, also has a major impact on energy metabolism, including mitochondrial function. The oncogene product p53, which has critical roles in DNA repair and the control of apoptosis, has recently been implicated in the balance between glycolysis and mitochondrial electron transport. On the other hand, the hypoxia response system, mediated by HIF-1 and the Von Hippe–Lindau factor (VHL), which directly regulates the expression of glycolytic enzymes, promotes angiogenesis and suppresses mitochondrial biogenesis, has also been found to regulate p53 activity. Also, AMP-activated protein kinase (AMPK) is a classical mediator of responses to metabolic stress, but recent studies have identified AMPK and its associated control mechanisms as contributors to the control of apoptosis and growth in tumor cells.

It appears from these examples that a deregulated cell signaling machinery may be a critical driver for the defects in the integration of metabolism, and cell survival and growth control in tumor cells. Later, we provide a brief overview of our current understanding of this integrated function. Figure 1 provides a summary overview of core cell signaling pathways and their target processes that provides a guide to the discussion.

The Akt Signaling System, a Survival Pathway that Functions to Facilitate Aerobic Glycolysis

Akt, also known as protein kinase B, is an oncogene that exhibits enhanced activity in numerous types of cancers. The Akt signaling pathway can be engaged by receptor tyrosine kinases, such as epidermal growth factor receptor and insulin-like growth factor receptor, which themselves frequently exhibit aberrant activity in transformed cells. The deranged Akt activity of cancer cells is responsible in part, for the uncontrolled cell proliferation, increased resistance to apoptosis, and altered cell metabolism, which is the characteristic of transformed cells (Kim et al. 2001; Bijur and Jope 2003; Majewski et al. 2004; Plas and Thompson 2005). Akt is not localized to the plasma membrane but is recruited there by the binding of the D3 lipid, phosphatidylinositol 3,4,5,trisphosphate (PIP3), which binds to a pleckstrin homology domain (PH domain) located in the amino-terminus of Akt (Manning and Cantley 2007). In turn, PIP3 is generated by the activation of phosphatidylinositol 3-kinase (PI3K). PI3K consists of a regulatory p85 and catalytic p110 subunits. Activated growth factor receptors and active Ras can recruit the p85 subunit and

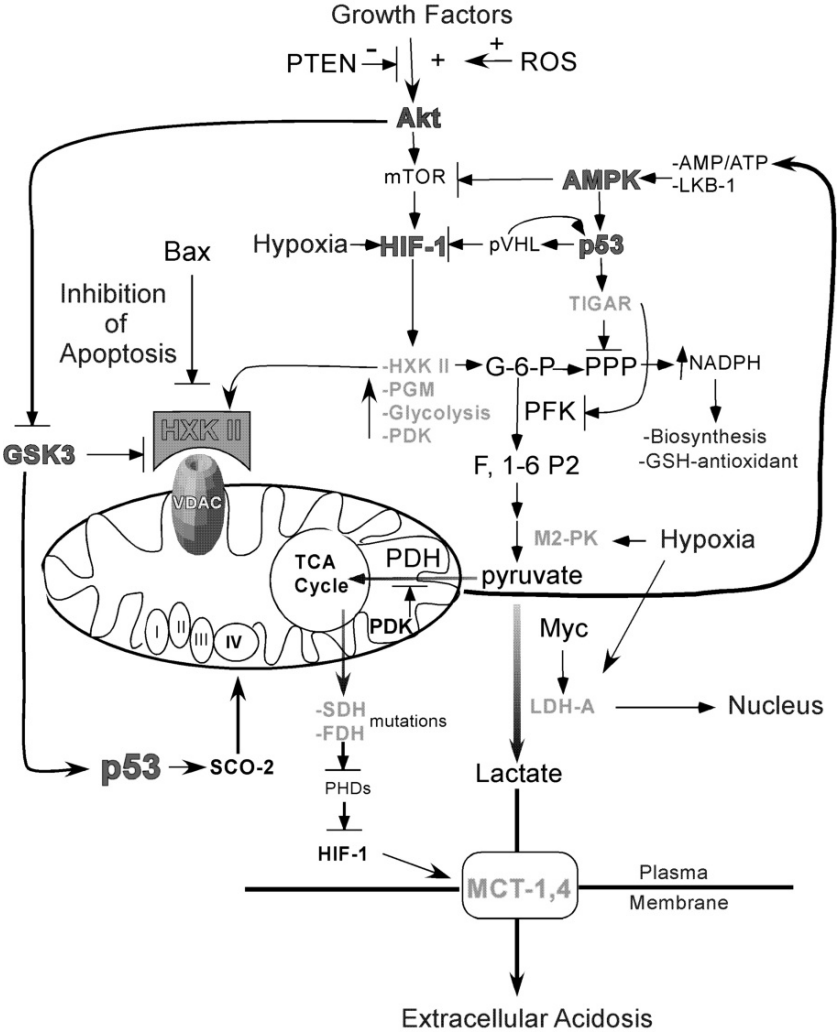

Fig. 1 Major connections between pathways of energy metabolism and apoptosis related to cellular signaling pathways in tumor cells

thus stimulate the p110 catalytic subunit that phosphorylates phosphoinositides at the D3 position to generate PIP3. Once PIP3 is bound to the PH domain of Akt, two subsequent phosphorylation events of Akt are required for its full activation. These phosphorylations are carried out by phosphoinositide-dependent kinase-1 at threonine 308, which is in the catalytic domain of Akt and by phosphoinositide-dependent kinase-2 at a serine residue (serine 473) in the carboxy-terminal region of Akt. In addition to increased activation, the PI3K-Akt pathway may also be stimulated inappropriately in cancer cells by dysfunction of PTEN (phosphatase and tensin

homologue deleted on chromosome 10), a lipid phosphatase (Cantley and Neel 1999). PTEN dephosphorylates PIP3, thereby dampening Akt activity. Thus a decrease in PTEN activity would cause a stimulation of Akt. Indeed, PTEN is a tumor suppressor that is inactivated in several sporadic and inherited tumor types that exhibit hyperactivation of Akt.

Akt Regulation of Hexokinase II in the Control of Energy Metabolism

Cancer cells with increased Akt activity exhibit an augmented uptake, utilization, and dependence on glucose. Akt triggers an increase in the transcription of the glucose transporter 1 (GLUT1) and stimulates its translocation to the plasma membrane from intracellular vesicles, where it mediates the uptake of glucose (Vander Heiden et al. 2001). From there, glucose enters the glycolytic pathway, where Akt also exerts an affirmative control over the activity of hexokinase, which catalyzes one of the rate-controlling steps of glycolysis.

Four major isoforms of hexokinase exist in mammalian cells (hexokinase I–IV), but many tumor cells abundantly express the hexokinase II isoform. The hexokinase I and II isoforms have the capacity to directly bind to the mitochondrial outer membrane, through the Voltage Dependent Anion Carrier, VDAC, an abundant outer mitochondrial membrane protein responsible for allowing the transport of most metabolites in and out of the mitochondria (Pastorino and Hoek 2003, 2008). In its localization at the mitochondria bound to VDAC, hexokinase gains preferential access to the ATP generated by the mitochondria through oxidative phosphorylation. In this way, the energy generated by oxidative phosphorylation is thought to be funneled to spike the glycolytic cascade (reviewed in Pedersen et al. 2002; Mathupala et al. 1997; Wilson 1995). However, the metabolic conditions under which such channeling of ATP would be beneficial for tumor cell metabolism remain unclear (see Pastorino and Hoek 2003 for a more detailed discussion). The hexokinase I and II isoforms possess a hydrophobic N-terminal sequence of 15 amino acids that has features compatible with an amphipathic α-helical structure (Xie and Wilson 1988; Sui and Wilson 1997). This N-terminal domain is essential and sufficient for mitochondrial binding of the protein. The isolated N-terminal peptide displaces hexokinase II bound to isolated mitochondria and hexokinase II could be specifically dislodged from the mitochondrial membrane in intact HeLa cells by expression of the N-terminal peptide (Pastorino et al. 2002).

Hexokinase binding to mitochondria may have other consequences that are relevant for the regulation of the enzyme activity. For instance, the sensitivity of the enzyme to regulators of glycolytic flux is affected by mitochondrial binding. Hexokinase II bound to mitochondria is less sensitive to inhibition by G-6-P (Wilson 2003; Mathupala et al. 2006). This would facilitate the accumulation of G6P in the cell to drive the pentose phosphate shunt and support the supply of metabolic intermediates for nucleotide synthesis and NADPH for oxidative stress

defenses. However, the conformational changes induced by very high levels of G-6-P promote the detachment of hexokinase II from the mitochondria. Alternatively, hexokinase binding may be important for the function of VDAC and its associated complex of proteins. There is considerable evidence that VDAC is required for the formation of complexes between the mitochondrial inner and outer membranes referred to as contact sites. Such contact sites can be discerned in electron microscopic studies both on isolated mitochondria, and on mitochondria in intact cells (Frey and Mannella 2000). Other proteins in the contact sites are thought to involve adenine nucleotide translocator and creatine kinase (Brdiczka et al. 1998). In addition, accessory proteins in the contact site complex may involve anti-apoptotic proteins of the Bcl-2 family that are known to interact with VDAC and ANT.

Akt controls the physiological effects of hexokinase II by influencing the localization of the enzyme at the mitochondria. Akt exerts its effect on mitochondrial binding of hexokinase II by influencing the phosphorylation state of VDAC. When VDAC is phosphorylated, hexokinase II is released from the mitochondria into the cytosol. However, Akt does not exert this control directly, but rather negatively controls the ability of GSK3β to phosphorylate VDAC (Pastorino et al. 2005). Akt is capable of phosphorylating GSK3β on serine 9, thereby inhibiting its activity. By contrast, when Akt activity is downregulated, GSK3β becomes stimulated, phosphorylating VDAC and thereby inducing the release of hexokinase II. GSK3β affects many other cellular events, ranging from metabolism to cell motility and cell differentiation. In addition, GSK3β responds to upstream signaling processes related to the Wnt signaling network. As a result the Akt-GSK3β axis functions as an important integration site for coordinating metabolic and proliferative control of the cell (Forde and Dale 2007).

Since Akt activity is upregulated in many cancers, where hexokinase II is abundant as well, a greater proportion of hexokinase II is bound to the mitochondria in transformed vs. normal cells. The increased production of G-6-P also enhances the uptake of glucose by the plasma membrane glucose transporters. The elevated levels of G-6-P can be shunted into the pentose phosphate pathway (PPP), where metabolic intermediates for DNA synthesis are generated (Wilson 2003; Boren et al. 2001; Burgering and Kops 2002). Additionally, the PPP is the predominant pathway responsible for the production of NADPH required for reductive biosynthesis and protection against oxidative stress. Importantly, all of these metabolic alterations provide the necessary biosynthetic environment required for rapidly proliferating cancer cells.

Intriguingly, not only can Akt activity enhance glycolysis, but glycolysis can stimulate Akt. Indeed it has been demonstrated in cells harboring mitochondrial DNA deletions that an increase in the level of NADH caused by respiratory deficiency and the subsequent reliance on glycolysis causes inactivation of PTEN through a redox mediated mechanism (Pelicano et al. 2006). The downregulation of PTEN then leads to Akt activation. In this way a positive feedback loop is set up in which the promotion of glycolysis by Akt through the increased uptake of glucose and binding of hexokinase to the mitochondria leads to a buildup of NADH that then inactivates PTEN, thereby stimulating Akt.

Akt Enhances Cell Survival Mechanisms through its Effects on Mitochondrial Binding of Hexokinase II

Akt has been recognized as an important mediator of cell survival pathways under conditions of stress. Akt effects on Hexokinase II binding to the mitochondrial outer membrane may serve as an important mechanism to suppress the mitochondrial contribution to apoptosis in tumor cells.

A large body of evidence indicates that VDAC is involved in the release of pro-apoptotic proteins from the mitochondrial intermembrane space in response to conditions that promote apoptosis. Several mechanisms may be involved. Disruption of the outer membrane is one obvious mechanism. Owing to the greater surface area of the inner mitochondrial membrane when compared with that of the outer mitochondrial membrane, excessive swelling of the mitochondrial matrix could result in rupture of the outer membrane and the consequent release of contents of the intermembrane space. Swelling and outer membrane disruption may result from opening of the permeability transition pore (PTP), which is thought to involve VDAC and ANT. The permeability transition is activated in response to a variety of noxic stimuli and PTP opening with subsequent mitochondrial swelling, depolarization, and outer membrane rupture does occur in some forms of apoptosis (Marchetti et al. 1996). Characteristically, the activation of PTP opening is inhibited by cyclosporin A, an effect mediated by the matrix protein cyclophilin D, which is presumed to interact with the matrix side of the ANT protein. Although the protein components in the PTP complex are similar to those involved in the formation of contact sites, the relationship between these structures remains poorly characterized. However, studies from our group (Pastorino et al. 2002) demonstrated that hexokinase II binding to isolated mitochondria suppressed PTP opening in response to various agents that activate the permeability transition.

Although its precise role in apoptosis remains controversial, VDAC appears to be involved as well in the release of intermembrane space proteins through a process that does not require inner membrane disruption and mitochondrial depolarization, where intermembrane space proteins are released into the cytosol in the absence of mitochondrial depolarization or overt signs of outer membrane rupture (Granville and Gottlieb 2003). Importantly, the release of intermembrane proteins through VDAC may be controlled by its interaction with pro- and anti-apoptotic proteins of the Bcl-2 family (Shimizu et al. 1999). These functions of VDAC in the regulation of the mitochondrial phase of apoptosis may provide an alternative view of the functional implications of mitochondrial binding of hexokinase II (Pastorino and Hoek 2008).

The binding of hexokinase II to mitochondria inhibits the ability of Bax to induce the release of cytochrome c from the mitochondrial intermembrane space (Pastorino et al. 2002; Vyssokikh et al. 2002). Treatment of isolated HeLa cell mitochondria with agents that promoted the detachment of hexokinase II from the mitochondria sensitized the mitochondria to Bax induced release of cytochrome c from the intermembrane space to the supernatant, while having no effect on the

inner mitochondrial membrane protein, cytochrome oxidase (Pastorino et al. 2002). These agents included not only G-6-P and the amphipathic compound, clotrimazole, but also the N-terminal oligopeptide of hexokinase II. This implies that the inhibitory effect of hexokinase II was not due solely to the occupation of the site that interacts with the N-terminal peptide, but must involve other regions of the protein. Addition of free hexokinase II to rat liver mitochondria, which have no endogenous hexokinase II, effectively prevented even high levels of Bax from binding to the mitochondria and promoting cytochrome c release. The N-terminus of hexokinase II was necessary for it to exhibit antagonistic activity toward Bax, as a 15 amino acid N-terminal truncated version of hexokinase II displayed no inhibition of Bax induced cytochrome c release from isolated rat liver mitochondria. Similar effects were evident in the intact cell. Treatment of intact HeLa cells with either 20 μM of clotrimazole or 100 μM of a cell permeable version of the hexokinase II N-terminal oligopeptide resulted in the release of endogenous hexokinase II from the mitochondrial to the cytosolic fraction. Moreover, the detachment of the hexokinase II was found to sensitize the cells to apoptosis. Indomethacin is a nonsteroidal antiinflammatory drug that has been demonstrated to bring about cell killing in colon carcinoma cells through its ability to promote the translocation and mitochondrial localization of Bax (Zhang et al. 2000). Pretreatment of HeLa cells with either clotrimazole or the N-terminal oligopeptide of hexokinase II markedly enhanced the apoptotic cell killing induced by indomethacin. Importantly, this was accompanied by an increase in the ability of Bax to bind to the mitochondria in clotrimazole and hexokinase II N-terminal oligopeptide pretreated cells exposed to indomethacin.

How hexokinase II inhibits the mitochondrial dysfunction brought about by proapoptotic proteins is unknown. Hexokinase II is presumed to bind to mitochondrial VDAC in the form of a tetramer, which would have a molecular of 400 kDa. Thus, it may interfere with Bax binding to VDAC by virtue of steric hindrance. This would suggest a model by which Bax (or other proapoptotic Bcl-2 family proteins) can compete with hexokinase II for binding to VDAC to promote outer membrane permeabilization, either through opening an outer membrane pore detached from the ANT, or by activating a permeability transition. Alternatively, we have suggested previously (Pastorino and Hoek 2003) that hexokinase II may contain a cryptic BH4 domain that could contribute to its antiapoptotic potential by competing with VDAC binding of the anti-apoptotic protein Bcl-x_L (Pastorino and Hoek 2008).

Akt, Control of Metabolism through mTOR and FOXO

Akt also exerts control over cell size and metabolic activity through its regulation of mTOR (Edinger and Thompson et al. 2002; Inoki et al. 2002; Hay 2005; Bhaskar and Hay 2007). The mammalian target of rapamycin (mTOR) is a serine–threonine kinase that controls cell growth by regulating protein synthesis of capped mRNAs. Capped mRNAs have structured 5′ untranslated regions that require the activity of the mTOR raptor complex (mTORC1) to be translated. Many proteins produced

from these capped mRNAs are involved in cell survival, proliferation, and metabolism. The mTOR raptor complex initiates the translation of capped mRNAs by stimulating the activity of the S6 ribosomal subunit kinase (S6K) and inhibiting the translational repressor, eukaryotic initiation factor binding protein, thereby lessening inhibition of eIF and initiating translation. Importantly, Akt positively regulates the activity of mTOR in the raptor complex by controlling the activity of the small GTPase named Rheb. Akt accomplishes this by phosphorylating and relieving the inhibition on Rheb exerted by the TSC1/TSC2 heterodimer, which is a GTPase activating protein (GAP) and thereby exerts a negative affect on Rheb activity. The importance of this pathway in the control of cell size is exemplified by the familial tumor syndrome, tuberous sclerosis complex, where TSC1 or TSC2 is mutated and inactivated. Thus TSC1 and TSC2 are considered tumor suppressors. However in this syndrome the tumors (hamartomas) that develop are benign, thereby indicating that the inactivation of the TSC1/TSC2 complex is adequate for deregulation of growth control but not attainment of transformation (Inoki et al. 2003; Li et al. 2004).

Akt also controls the activity of the FOXO (F-box protein, subclass O) transcription factors (Burgering and Kops 2002; Zhu et al. 2004). Activation of Akt by growth factors results in the sequestration of FOXOs in the cytosol and their subsequent degradation by the proteasomal pathway. Dephosphorylation of FOXO transcription factors causes their translocation to the nucleus. The FOXO transcription factors upregulate the synthesis of proteins necessary for a ramping up of oxidative metabolism, such as superoxide dismutase 3. The increased rate of oxidative metabolism and formation of reactive oxygen species (ROS) generated require a pause in the cell cycle that is also mediated by FOXOs through their stimulation of insulin-like growth factor binding protein 1, p21, and p27, inhibitors of cell cycle progression. In a worse case scenario, FOXOs also stimulate the expression of pro-apoptotic proteins such as Bim, Bax, and Fas ligand, most likely required when cell injury caused by the increased oxidative metabolism provokes irreparable cell damage (Modur et al. 2002; Sunters et al. 2003). From this it can be appreciated that inappropriate stimulation of Akt, as seen in transformed cells, will lead to an inactivation of FOXO transcription factors. This will then force the cell to rely more on glycolysis, suppress apoptosis, and promote cell cycle progression even in the presence of DNA damage.

AMPK-Survival Under Conditions of Nutrient Scarcity

The rapid growth of cancer cells and the disordered angiogenesis that occurs in tumors results in the development of hypoxia in the tumor's interior regions and severe limitations in nutrient availability. Under stressful conditions such as these, the AMP dependent protein kinase is activated. AMPK is a heterotrimer composed of α, β, and γ subunits (Rutter et al. 2003; Hardie 2005). AMPK activation is triggered by a decrease in the AMP/ATP ratio as would occur under hypoxic and nutrient limiting conditions. In addition, AMPK activity responds to hormones (such as leptin and adiponectin) that regulate the balance of fat and glucose

metabolism to facilitate its capacity of the organism to handle periods of food scarcity (Marshall 2006). Activation of AMPK reconfigures cell metabolism from one of an anabolic, energy utilization intensive state, to a catabolic, energy conserving state. It brings this about by inhibiting anabolic pathways like fatty acid synthesis and activating energy generating pathways such as fatty acid oxidation (Tomita et al. 2005). In this way, activation of AMPK prepares the cell for a prolonged siege of austere environmental conditions and enables it to survive until more favorable circumstances prevail. Since protein synthesis is an energy and nutrient intensive process, an important component of the energy and nutrient conserving strategy initiated by AMPK is inhibition of mTOR.

The GAP activity of the tuberous sclerosis complex (TSC1/TSC2) is stimulated by AMPK through its phosphorylation of tuberin (TSC2) (Hardie 2004; Inoki et al. 2006). This causes an inhibition of the small GTPase Rheb, which results in a stifling of mTORC1 activation. This action of AMPK antagonizes the Akt mediated activation of mTORC1. This places mTORC1 in a central position of mediating the input from the Akt pathway that promotes anabolic metabolism necessary for rapidly proliferating cancer cells and input from AMPK, which promotes energy and nutrient conservation and an inhibition of cell division. The input from these two pathways on mTORC1 activity is integrated by TSC2. AMPK phosphorylates several sites within TSC2 that is inhibitory to its GAP activity while Akt phosphorylation of TSC2 stimulates TSC2 GAP activity, thus positioning these two kinases in antagonistic roles.

One of the upstream kinases that phosphorylate AMPK is LKB1 (Woods et al. 2003; Imai et al. 2006). LKB1 phosphorylates AMPK on threonine 172 in its catalytic α subunit. This is attended by allosteric activation by AMP, which enhances AMPK activity by making it a poorer substrate for dephosphorylation. LKB1 is a tumor suppressor. LKB1 mutations that cause loss of its kinase activity result in Peutz–Jeghers syndrome, an autosomal-dominant disease characterized by hamartomas, as in tuberous sclerosis caused by inactivation of the TCS1/TSC2 complex. Both conditions would allow unchecked activation of mTORC1, thus reinforcing the role of mTORC1 in the control of cell metabolism and size.

Paradoxically, the activation of AMPK may be necessary for tumors to progress, at least in the short term. As stated earlier, the rapid proliferation of cancer cells frequently outpaces the ability to provide them with adequate oxygen and nutrients. In providing a mechanism to get through these limiting conditions, AMPK eventually contributes to the survival of the tumor cells and their expansion when angiogenesis again enables the delivery of adequate oxygen and nutrients for cell growth and division (Kato et al. 2002; Carling 2004; Luo et al. 2005; Shaw 2006). In this way, activation of AMPK is analogous to the inhibition of cell cycle progression mediated by p53 in response to DNA damage. In this instance, p53 inhibits cell cycle progression by promoting the synthesis of p21 and p27. In this way p53 enables the cell to pause for DNA repair, otherwise irreparable DNA damage would occur, thus endangering the viability of the cell. In an analogous manner, AMPK activation manages the crisis of energy shortage, enabling the cell to survive until the establishment of more favorable conditions by increased

angiogenesis. Indeed, AMPK phosphorylates and activates p53, thus engaging a p53 dependent metabolic checkpoint (Jones et al. 2005). AMPK phosphorylates p53 on serine 15 and initiates an AMPK dependent cell cycle arrest. The AMPK dependent phosphorylation and activation of p53 occurs in response to glucose deprivation. When glucose supplies are restored, the cell reenters the cell cycle. If the glucose deprivation is persistent, then the cell enters a senescent state. This places AMPK in a critical position of balancing cell proliferation with nutrient availability. Thus under circumstances where nutrients are limiting, AMPK parks the cell in a standby mode where it conserves energy and can await for improvement in the supply of nutrients to rapidly reenter the cell cycle.

The Role of P53 in Cancer Cell Metabolism

The interaction of AMPK and p53 places p53 at a critical node of cell metabolism. Indeed, apart from its well established role as a regulator of the cell cycle and apoptosis, p53 has recently emerged as a vital regulator of cellular energy generation. This is exemplified by the control exerted by p53 on the transcription of a key regulator of oxidative phosphorylation, synthesis of cytochrome c oxidase 2 (SCO2) (Matoba et al. 2006; Kruse and Gu 2006; Assaily and Benchimol 2006). The expression of the assembly protein, SCO2, is upregulated in a p53 dependent manner. SCO2 is essential for the assembly of the cytochrome c oxidase complex of the respiratory chain. A decrease of SCO2 levels causes an impairment of proper COX assembly and results in defective mitochondrial aerobic respiration. COX IV is a multimeric protein complex located at the inner mitochondrial membrane. COX IV mediates the transfer of electrons from reduced cytochrome c to molecular oxygen. The transfer of electrons to COX IV induces a conformational change in its structure that results in the migration of protons across the inner mitochondrial membrane, thus creating and maintaining the mitochondrial proton electrochemical gradient, which is then used for ATP synthesis. Inactivation of p53 is the most common mutation found in cancers. This could be a possible explanation, in part, for the increased reliance of cancer cells on glycolysis for energy production. Indeed, disruption of the SCO2 gene in human cancer cells expressing wild-type p53 replicated the reconfiguration of cell metabolism toward a dependence on glycolysis (Assaily and Benchimol 2006). The fact that p53 deficiency can cause a loss of mitochondrial respiration and increase reliance on glycolysis may provide a partial explanation for the Warburg effect. This is illustrated in mice with homozygous deletion of p53, where there is a decrease in aerobic respiration and increased dependence on glycolysis, manifested as a decline in oxygen consumption, increase in lactate production and exercise intolerance.

The rechanneling of glucose toward glycolysis by haploinsufficiency of p53 may also be mediated by a reduction in the expression of TIGAR (TP53-induced glycolysis and apoptosis regulator). Like hexokinase, phosphofructokinase-1 is one of the rate-controlling enzymes in the glycolytic pathway, which controls the

distribution of carbon flux between the pentose phosphate shunt and the glycolytic cascade itself. The metabolite, β-D-fructose 2,6 bisphosphate (F-2,6-BP), is a potent allosteric activator of phosphofructokinase-1, thus increasing glycolytic flux. Fructose 2,6 bisphosphate is formed by the phosphorylation of fructose-6-phosphate. The reaction is catalyzed by phosphofructokinase-2 (PFK2) (Minchenko et al. 2002; Chesney 2006). By contrast, fructose bisphosphatase 2 (FBPase 2) hydrolyzes F-2,6-BP to fructose-6-phosphate, thereby dampening glycolysis. Interestingly, the PFK2 and FBPase 2 activities are present on the same enzyme. Intracellular levels of F-2,6-BP are controlled by a family of bifunctional enzymes termed 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB1–4) encoded by four genes. The PFKFB3 isoform is frequently expressed in human tumors. TIGAR possesses a bisphosphatase activity and displays functional and sequence similarities to the bisphosphatase domain of PFKFB 2. In keeping with this bisphosphatase activity, expression of TIGAR was found to decrease glycolytic flux at the point of phosphofructokinase. This results in an accumulation of glucose-6-phosphate. The glucose-6-phosphate thus formed is shunted into the pentose phosphate pathway. In this way, TIGAR stimulates the PPP at the expense of glycolysis. In doing so, TIGAR increases the production of NADPH and intermediate metabolites such as ribose-5-phosphate used for DNA biosynthesis. NADPH is utilized as a reducing equivalent in several biosynthetic reactions that are highly active and required in proliferating cells. Additionally, NADPH is necessary for the synthesis of reduced glutathione (GSH), a critical antioxidant. The induction of TIGAR expression by p53 may thus serve a protective function against oxidative stress. As outlined earlier, p53 also stimulates the cellular capacity for oxidative phosphorylation by enhancing the synthesis of SCO2, thereby bringing about an increase of cytochrome oxidase activity. This may further help suppress the formation of reactive oxygen species. The mitochondrial respiratory chain is a major source of ROS, but this occurs largely through electron transport complexes I and III that function upstream of cytochrome oxidase. An excess of electron supply to the respiratory chain that exceeds the capacity of cytochrome oxidase to handle it would therefore stimulate ROS production. If the cytochrome oxidase activity is enhanced by p53/TIGAR-mediated SCO2 production, this stimulation of aerobic respiration by p53 may therefore result in a decrease of ROS production. Since TIGAR shunts G-6-P to the PPP, resulting in an increased production of NADPH and the antioxidant GSH, the upregulation of TIGAR by p53 may coordinate with the increased flux capacity of mitochondrial respiration to suppress cellular oxidative stress. A reduction in this effect would enhance ROS formation in cancer cells.

Glycolysis can also be controlled by p53 through its regulation of two glycolytic enzymes, hexokinase II and phosphoglycerate mutase (PGM). The expression of PGM is downregulated by wild-type p53 and increased by loss or mutation of p53 (Kim and Dang 2005, 2006; Ma et al. 2007). Similarly, expression of mutant but not wild-type p53 stimulates the expression of hexokinase II, consistent with the identification of two functional p53 response elements in the hexokinase II promoter (Mathupala et al. 1997). These observations are also consistent with wild-type p53 promoting mitochondrial oxidative phosphorylation. By contrast, mutation and

inactivation of p53 results in a decrease of oxidative phosphorylation through a diminished synthesis of cytochrome oxidase and increased production of the glycolytic enzymes, hexokinase II and PGM, which in combination may account, in part, for the dependence of tumor cells on glycolytic energy metabolism.

In addition to its role in promoting oxidative metabolism and downregulating glycolysis, there are several observations that p53 directly impacts mitochondrial function and structural integrity in the context of apoptosis. Several apoptotic stimuli provoke a rapid translocation of p53 to the outer mitochondrial membrane (Moll et al. 2006). Since p53 lacks mitochondrial-targeting sequences, the signal that mediates the localization of p53 to the mitochondria remains unidentified. Mitochondrial p53 can permeabilize the outer mitochondrial membrane without the need for other interacting proteins. However experimental observations indicate that GSK3β binds to p53 and elevates its ability to promote the release of intermembrane space proteins (Watcharasit et al. 2003). In conjunction with this, wild-type but not mutant p53 can bind to and antagonize the antiapoptotic actions of Mcl-1, Bcl-2, and Bcl-X_L, thus inflicting a double hit on mitochondrial outer membrane permeabilization. The ability of p53 to bind and antagonize the actions of the antiapoptotic proteins imbues p53 with the ability to indirectly activate the pro-apoptotic proteins Bak and Bax. For example, upon apoptotic stimuli, p53 translocates to the outer mitochondrial membrane and liberates pro-apoptotic Bak from its sequestration by Mcl-1. These direct damaging effects of p53 on the mitochondria are accompanied by p53s' ability to induce the synthesis of pro-apoptotic proteins such as Bax and PUMA though its transcriptional capabilities (Yu et al. 2001).

It appears that p53 plays a dual role in mitochondrial function. When there is little or no exogenous stress impinging on the cell, p53 promotes mitochondrial oxidative phosphorylation. However when p53 is stimulated above a threshold level, it provokes irreparable mitochondrial injury, with a resultant loss in the ability of the mitochondria to meet the energetic demands of the cell. The energetic failure of the mitochondria is accompanied by the release of apoptotic proteins from the mitochondrial outer membrane, thereby mediating cell death. However when p53 is mutated and dysfunctional, there is a decrease in mitochondrial oxidative phosphorylation owing to inadequate assembly of cytochrome oxidase and a concomitant augmentation of glycolysis, promoted by the upregulation of the glycolytic enzymes hexokinase II and PGM, and the downregulation of TIGAR, both effects promoting an increase in glycolytic flux. Therefore when p53 is functioning normally, it promotes mitochondrial oxidative phosphorylation and resistance to oxidative stress by upregulating TIGAR. However when the cell suffers irreparable injury, p53 is responsible for inflicting mitochondrial damage, which leads to cell demise. By contrast, mutated and dysfunctional p53 promotes a decrease in mitochondrial oxidative phosphorylation, an augmentation of glycolysis and may account in part for the Warburg effect.

The involvement of p53 in the regulation of energy metabolism is highlighted by its stabilization and transactivation by von Hippel–Lindau tumor suppressor (Roe et al. 2006). Inactivation of the von Hippel–Lindau tumor suppressor gene is linked to hereditary cancer syndromes such as renal cell carcinoma. This is thought to be

mediated by a stimulation in the activation of HIF-1 (hypoxia inducible factor), leading to a boost in glycolysis. However recent observations indicate that pVHL can also regulate p53 activity and levels. The pVHL protein binds to and stabilizes p53 and in conjunction with a serine kinase and acetyltransferase, enhances its transcriptional activity. In the absence of pVHL, p53 levels and activities are diminished. As outlined earlier, p53 enhances mitochondrial oxidative metabolism and mutations or absence of p53 stimulates glycolysis. Therefore the increased dependence of VHL deficient cells on glycolysis maybe mediated in part by a diminution of p53 activity. Additionally, p53 is linked to HIF-1 activity by regulating its degradation.

Dysregulation of HIF-1 in Cancer Cells and its Impact on Cell Metabolism

HIF-1 is a heterodimer composed of HIF-1β and HIF1α. The HIF-1β subunit is stable and constitutively expressed (Semenza 2007). In contrast, HIF-1α is unstable, rapidly degraded and subject to extensive regulation. The level of HIF1α is dependent on the degree of oxygenation. Prolines 402 and 564 of HIF-1α lie in the oxygen-dependent degradation (ODD) domain and are hydroxylated by the prolyl hydroxylase-domain enzymes, PHD1–3. PHDs require oxygen for their activity; when oxygen levels are limiting, their activity is diminished. Under oxygen replete conditions, the hydroxylation of HIF-1α by the PHDs promotes the interaction of HIF-1α with pVHL. VHL exists in a multimeric complex along with elongin C, B, Cul2, and Rbx, which interact with VHL through its α domain. The β domain of VHL interacts with the hydroxylated HIF-1α and targets it for proteasomal degradation. In the absence of oxygen, PHDs cannot promote the hydroxylation of HIF-1α and therefore pVHL is not able to bind to HIF-1α, resulting in a decrease of HIF-1α degradation and it subsequent accumulation. By contrast in the absence of pVHL, even when HIF-1α is hydroxylated, it is not ubiquitinated and degraded insufficiently, accounting in part for the enhanced glycolysis seen in renal-cell carcinoma, where the VHL gene is inactivated.

During hypoxia or in the absence of pVHL, HIF-1α translocates to the nucleus, where it heterodimerizes with HIF-1β and stimulates the expression of genes containing HIF responsive elements (HREs) in their promoters (Zuurbier et al. 2005; Sun et al. 2006). The activation of HIF-1 drives the transcription of genes that promote angiogenesis, such as VEGF and angiopoetin-2. Concurrently, HIF-1 increases the synthesis of the glucose transporter, GLUT-1, and the enzymes of glycolysis, particularly hexokinase and PGM. In this manner, HIF-1 enables the cell to endure hypoxia through the enhanced rate of glycolysis until a greater supply of oxygen can be delivered by means of increased angiogenesis.

An increase in HIF-1α levels can also be stimulated independent of oxygen. Activation of growth factor receptors induces HIF-1α expression. However in contrast to the hypoxic regulation of HIF-1α, the elevated levels of HIF-1α caused

by growth factors is due to an increase in synthesis rather than by a decrease in degradation, as seen in hypoxia. Heregulin stimulation of breast cancer cells was demonstrated to cause an increase in HIF-1α synthesis that was prevented by pretreatment with rapamycin. This observation indicates that mTORC1 can mediate an increase in the cap-dependent translation of HIF-1α mRNA (Choo and Blenis 2006). Interestingly, this places HIF-1α regulation downstream of Akt. Akt inhibits the TSC1/TSC2 complex, thereby stimulating mTORC1, which in turn increases the synthesis of HIF-1α. The increased production of HIF-1α will then lead to the synthesis of HIF-1 dependent genes such as the glycolytic enzymes and an increase in glycolysis. Additionally, activation of the RAF-MEK-ERK signaling pathway by growth factors stimulates HIF-1 transactivation via the phosphorylation by ERK of the transcriptional coactivator p300, which interacts with the HIF-1 transactivation domain.

Significantly there is a point of divergence between the growth-factor-stimulated increase in HIF-1 and that of hypoxia in that hypoxia initiates a negative feedback to stifle HIF-1 activity. This is mediated in part by REDD1 and 2 that are transcriptionally induced by HIF-1 during hypoxia but not by growth factors (Reiling and Hafen 2004; Schwarzer et al. 2005). REDD1 and 2 are thought to inhibit mTORC1 by activating the TSC1/TSC2 complex, although the mechanism by which this is accomplished is unknown. Additionally, hypoxic conditions promote the binding of promyelocytic leukemia (PML) tumor suppressor that interacts with mTORC1 to prevent its association with Rheb (Bernardi et al. 2006). Therefore the activation of HIF-1 by hypoxia is constrained, whereas that induced by growth factors is less self limiting. Such observations have implications for HIF-1 activation in the setting of transformed cells. The activity of Akt and mitogenic pathways such as ERK are frequently heightened and dysregulated in cancer cells. This would result in a stimulation of HIF-1 activity that would not experience the negative feedback that constrains HIF-1 stimulation during hypoxia. Concomitantly, HIF-1 target genes include those that encode growth factors such as IGF-2 and TGF-α, thus exacerbating HIF-1 stimulation.

The activation of HIF-1 by deregulated Akt would result in an increase in glycolytic enzymes and flux through the glycolytic pathway even in the presence of adequate oxygen supplies. This is compounded by the ability of HIF-1 to suppress mitochondrial oxidative phosphorylation. Indeed, HIF-1 stimulates the synthesis of pyruvate dehydrogenase kinase-1 (PDK-1) (Papandreou et al. 2006; Kim et al. 2006). Phosphorylation of the catalytic subunit of pyruvate dehydrogenase (PDH) by PDK inactivates the enzyme. Pyruvate is the end product of glycolysis and under conditions of oxidative metabolism is taken up into the mitochondria, where PDH converts it into acetyl-CoA, which then enters the TCA cycle. The conversion of pyruvate to acetyl-CoA is irreversible and therefore a critical control point in the interface between glycolysis and oxidative phosphorylation. By inhibiting PDH through the induction of PDK, HIF-1 reconfigures the metabolism of the cell away from oxidative phosphorylation and toward a reliance on glycolysis. Instead of pryruvate being converted to acetyl-CoA for utilization in the TCA cycle, it is converted to lactate by lactate dehydrogenase (LDH), which HIF-1 also upregulates. This may partly explain the characteristic feature of cancer cells

producing lactate even under oxygenated conditions. The lactate produced is transported into the extracellular space by the monocarboxylate transporter (MCT1) that itself is induced by HIF-1 (Fang et al. 2006). The conversion of pryruvate to lactate enables the regeneration of NAD^+, which is required as a cofactor for the glycolytic enzyme glyceraldehydephosphate dehydrogenase, where it is reduced to NADH during the oxidation of glyceraldehydephosphate to bisphosphoglycerate.

It may be feasible to exploit as a therapeutic target, the reconfiguration of cancer cell metabolism mediated by HIF-1. It was demonstrated that dichloroacetate (DCA), an inhibitor of PDK, shifted the metabolism of cancer cells from glycolysis to oxidative metabolism of glucose (Bonnet et al. 2007). Significantly, DCA has minimal effects on normal cells that already rely on oxidative metabolism. The increase in oxidative phosphorylation seen in cancer cells treated with DCA is accompanied by an elevation in the production of reactive oxygen species (ROS) and decrease in mitochondrial membrane potential. The increased oxidative metabolism and elevated levels of ROS trigger a cascade of mitochondrial damage starting with complex I of the electron transport chain. In turn, complex I deficiency leads to a decrease in the mitochondrial membrane potential and opening of the mitochondrial permeability transition pore. The increase of ROS production also triggers an upregulation and opening of a potassium channel (Kv1.5) that promotes a decrease of cytosolic potassium, which contributes to induction of apoptosis by assisting in activation of caspases.

In addition to inhibiting mitochondrial oxidative phosphorylation, HIF-1 is capable of inhibiting mitochondrial biogenesis and therefore decreasing the mitochondrial mass of cancer cells (Zhang et al. 2007). It was recently revealed that C-MYC can regulate mitochondrial mass and oxygen consumption partly through increasing the expression of the transcriptional coactivator PGC-1β. By contrast HIF-1 negatively regulates the level of C-MYC and therefore of PGC-1β by inhibiting C-MYC transcriptional activity through an increased expression of MXI-1. MXI-1 competes with MAX for binding to C-MYC. C-MYC:MAX heterodimers activate transcription of C-MYC target genes whereas MYC:MXI-1 heterodimers repress expression. Also HIF-1 was demonstrated to mediate degradation of C-MYC through a proteasome-dependent mechanism. Therefore both of these mechanisms contribute to the decrease of PGC-1β, and resulting diminished mitochondrial biogenesis and mass mediated by activation of HIF-1.

The importance of HIF-1 in mediating the alterations in metabolism of cancer cells is also illustrated by the tumor suppressors succinate dehydrogenase and fumarate hydratase (King et al. 2006). Succinate dehydrogenase (SDH) is part of complex II of the electron transport chain in addition to being a TCA cycle enzyme that converts succinate to fumarate. Fumarate hydratase (FH) catalyzes the subsequent step of the TCA cycle and converts fumarate to malate. Mutations of the SDHB,C, and D subunits of succinate dehydrogenase are associated with the cancer syndrome of hereditary paraganglioma (HPGL). Mutations of the SDH genes follow the two hit sequence characteristic of tumor suppressors and are accompanied by a loss of heterozygosity. SDH mutations are also encountered in

sporadic paragangliomas and in pheochromocytomas. Mutations of the FH gene are associated with hereditary leiomyomatosis and renal cell carcinoma (HLRCC).

Loss of SDH and FH activity cause the condition known as pseudohypoxia, where the HIF-1 pathway is activated even in the presence of adequate oxygenation (MacKenzie et al. 2007). The biochemical basis of pseudohypoxia is the accumulation of succinate and fumarate in the mitochondrial matrix in SDH and FH deficiency, respectively. Succinate is transported out of the mitochondrial matrix by the dicarboxylate carrier and into the cytosol, where it can inhibit the activity of the PHDs. PHDs catalyze the conversion of a prolyl residue, molecular oxygen, and α-ketoglutarate to hydroxyl-prolyl, carbon dioxide, and succinate. Therefore succinate is the end product of PHD activity and as such can inhibit its activity. When PHD activity is repressed, HIF-1α is not hydroxylated and therefore cannot be bound by pVHL and ubiquitinylated for proteasomal degradation. This leads to an increase in HIF-1α levels even in the presence of oxygen. Similarly, fumarate, being structurally similar to succinate, inhibits the activity of PHDs and leads to a stabilization of HIF-1 and its subsequent transactivation of angiogenic and glycolytic genes. Fumarate is not a substrate for the dicarboxylate carrier and is not readily transported across the mitochondrial membrane. However, matrix accumulation of fumarate would effectively inhibit SDH activity, resulting in an effect that would be similar to that of a direct loss of SDH. Also, fumarate can be generated in the cytosol, e.g., through argininosuccinate lyase (an enzyme involved in processing citrulline) or adenylosuccinate lyase (which is part of the purine nucleotide synthesis pathway).

Pyruvate Kinase as a Regulator of Glycolytic Flux and Apoptosis in Tumor Cells

Pyruvate kinase is another rate-controlling enzyme in glycolysis, catalyzing one of the ATP producing reactions in the glycolytic cascade and providing pyruvate, which can be transported into the mitochondria for oxidation, reduced to form lactate, or converted to other metabolites. Four different isoforms of pyruvate kinase exist in humans, with tissue specific expression, designated L, R, M1, and M2. The M2-PK isoform is normally present in early embryonic development and is gradually replaced by the tissue-specific isoforms (Hardt et al. 2004; Mazurek et al. 2005). However, in many tumor cells, the M2-PK isoform is overexpressed and its presence in serum or fecal matter in recent years has been used as a biomarker for colorectal or gastrointestinal cancer, or other tumors (Kumar et al. 2007). There is evidence that M2-PK expression is enhanced by hypoxia, although the underlying mechanisms may not be entirely dependent on HIF-1 (Discher et al. 1998). Despite the high levels of expression of M2-PK in tumors, the activity of the enzyme is subject to regulation. M2-PK occurs either as a dimer or in tetrameric form. Only the tetramer is enzymatically active and capable of interacting with a complex of glycolytic enzymes. The interconversion between the dimeric and

tetrameric forms is affected by multiple factors. Activation is stimulated by the glycolytic intermediate fructose-1,6,-bisphosphate (Dombrauckas et al. 2005), which accumulates under conditions where phosphofructokinase-1 is active. Therefore, the activity of the M2-PK is intimately connected to the regulation of PFK, which, as indicated earlier, depends on the levels of fructose-2,6,-bisphosphate and the activity of TIGAR. However, the dimeric form of M2-PK is stabilized by tyrosine phosphorylation mediated by the src oncogene, among other factors (Mazurek et al. 2002). In addition, in a recent study, Christofk et al. (2008a) reported that M2-PK binds phosphotyrosine peptides in competition with fructose-bisphosphate. Therefore, a stimulation of protein tyrosine phosphorylation (e.g., in response to growth factor receptor or src activation) may displace fructose-bisphosphate and decrease pyruvate kinase activity in tumor cells. Importantly, tumor cells expressing a mutant form of M2-PK that is not capable of phosphotyrosine binding, or cells in which M2-PK was replaced by the M1-PK isoform, which is not sensitive to these regulatory features, lack the specific enhancement of aerobic glycolysis and lactate formation characteristic of the Warburg effect and when injected into susceptible mice they had a markedly reduced tumor size (Christofk et al., 2008b). M2-PK binds to microtubules and phospholipid. Mazurek et al. have demonstrated that the rate of glycolysis can be limited by the predominance of dimeric M2-PK, with the accumulation of glycolytic intermediates upstream of the pyruvate kinase step (Mazurek et al. 2002, 2005). These authors suggest that this condition may be important for the supply of metabolic intermediates for biosynthetic purposes, e.g., through PPP metabolites, or using triose phosphate intermediates of glycolysis (Mazurek et al. 2002, Christofk et al. 2008b). However, under these conditions the utility of the glycolytic pathway to meet cellular energy demand would be restricted, making the cell more dependent on the oxidation of mitochondrial substrates, notably glutamine. How the balance between these pathways is controlled at the level of the pyruvate kinase step and how this balance is affected by the metabolic state of the tumor, i.e., its response to the supply of substrate and oxygen remains insufficiently characterized.

This is probably the place to insert a brief mention of the recent work by Cantley and coworkers (Christofk et al. 2008) on M2-PK and its role in cancer energy metabolism. Interestingly, in a recent study (Stetak et al. 2007) it was demonstrated that M2-PK itself can be translocated to the nucleus and exert a pro-apoptotic effect that bypasses the mitochondrial pathway of apoptosis and is independent of caspase-3 and Bcl-2 family proteins. Importantly, the pro-apoptotic effect was specific for the M2-isoform and enzymatic activity of M2-PK was not required for these functions. To what extent this nuclear translocation is involved in regulating the balance between the metabolic functions and the control of apoptosis in the tumor is not yet clear. However, these observations emphasize the point that enzymes that are known for their metabolic functions may have other effects that do not require their enzymatic activities, but may affect the balance of energy metabolism and cell survival.

Lactate Dehydrogenase, the Terminal Enzyme of Anaerobic Glycolysis, is Critical for Cancer Cell Metabolism, Proliferation, and Survival

Lactate Dehydrogenase is a tetrameric enzyme composed of the subunits LDH-A and LDH-B in combinations that result in five isoforms (Fantin et al. 2006). The conversion of pyruvate to lactate is reversible, but LDH-A kinetically favors the production of lactate under anaerobic conditions in normal cells and is the isoform found overexpressed in transformed cells. The conversion of pyruvate to lactate oxidizes cytosolic NADH, resulting in the regeneration of NAD^+ required for sustained glycolytic flux through the glyceraldehyde phosphate dehydrogenase step. Under aerobic conditions, cytosolic NADH is oxidized by a hydrogen shuttle system, predominantly the malate-aspartate shuttle, which effectively uses these metabolites as intermediate carriers of the reducing power of NADH to transfer the electrons to mitochondrial NADH for oxidation by the mitochondrial respiratory chain. Also, under these conditions the pyruvate generated by glycolysis is transported into the mitochondria, where it is funneled into the TCA cycle generating NADH in the matrix. NADH oxidation by the mitochondrial respiratory chain then pumps protons across the mitochondrial inner membrane, thereby establishing the proton electrochemical gradient that drives ATP synthesis. However, under hypoxic conditions, electron transport is impaired and mitochondrial NADH is not reoxidized, thereby blocking the operation of the malate-aspartate shuttle. Oxidized NAD^+ in the cytosol required for continued glycolytic flux must be supplied by the conversion of pyruvate to lactate by LDH-A, without which glycolysis would grind to a halt.

Intriguingly in transformed cells, even under aerobic conditions, LDH-A is highly active (Fantin et al. 2006; Shim et al. 1997). As outlined earlier, this is due in part to the decrease in mitochondrial oxidative phosphorylation characteristic of cancer cells. However cancer cells also exhibit a robust increase in the levels of LDH-A. This is thought to be due to the increased activation of HIF-1 seen in cancer cells and also in part to the oncogene Myc (Shim et al. 1997), although alternative hypoxia response mechanisms may contribute (Discher et al. 1998). Myc, which participates in the regulation of cell proliferation, transactivates the LDH-A promoter and causes an increase of LDH-A levels. Suppression of LDH-A expression mitigated the ability of Myc to transform cells to an anchorage-independent neoplastic phenotype, suggesting that LDH-A activity is necessary for cancer cell growth and survival. Unfortunately, the mechanism by which this is accomplished is unknown. However it has been demonstrated that knock down of LDH-A stimulated oxidative phosphorylation in a subset of tumor cell lines and diminished the elevated mitochondrial proton electrochemical gradient characteristic of cancer cells. In contrast to the transformed parental cells, cells in which the expression of LDH-A was suppressed grew normally under normoxia but exhibited severe impairment of growth under hypoxia, thus establishing that LDH-A activity is necessary for tumor cell survival and proliferation under oxygen limiting

conditions. Moreover, the increased LDH-A activity may contribute, at least in part, to the inhibition of oxidative phosphorylation as a result of competition for NADH with malate dehydrogenase, which drives the cytosolic component of the malate-aspartate shuttle. In fact, there is evidence that the activity of these hydrogen shuttle mechanisms are reduced in tumor cells (Mazurek et al. 1997).

The rapid generation and accumulation of lactic acid could decrease cytoplasmic pH, which would impair the efficiency of the mitochondrial respiratory chain. Indeed, lactate formation would be detrimental to highly glycolytic cancer cells if not for the concomitant upregulation of the proton linked monocarboxylate transporter (MCT1 and 4), responsible for the export of lactate from the cytosol into the extracellular space. The MCT is a reversible symporter that carries lactate and a proton in tandem. The increased rate of lactate production, through the enhanced expression of LDH-A and MCT in the cancer cells, results in a net export of lactic acid from the cytosol into the extracellular space. In fact, the acidic extracellular environment caused by the discharge of lactic acid into the intracellular space of the tumor may assist the expansion of the tumor by decreasing the viability of the normal cells that surround it. In addition, lactic-acid accumulation may be deleterious to cells such as T cells that themselves rely on glycolysis and lactic acid secretion for energy supply (Fischer et al. 2007).

Of course, the maintenance of pH balance in the tissue surrounding the tumor is not solely determined by the H^+ cotransporters such as those involved in lactate transport, but is also determined by the activity of a pH regulating ion transporters in the plasma membrane, notably the Na^+/H^+ transporter and the bicarbonate transporters (Casey 2006). Indeed, a recent study (Provent et al. 2007) noted that the distribution of extracellular lactate and low pH in a solid tumor was not co-incident.

In addition to its role in metabolism, LDH-A possesses an ability to destabilize the DNA helix (Calissano et al. 1985; Cattaneo et al. 1985; Williams et al. 1985). The ability of LDH-A to bind single stranded DNA is prevented by NADH, which is a substrate of LDH-A, thus suggesting some regulatory interaction between the ability of LDH-A to bind single stranded DNA and its metabolic activity. The ability of LDH-A also appears to be regulated by tyrosine phosphorylation. Indeed, LDH-A was found to be a substrate of the epidermal growth factor receptor. Furthermore, nerve growth factor restrains the binding activity of LDH-A to single stranded DNA. However, the implications of these observations in the context of the elevated levels of LDH-A found in transformed cells is presently uncharacterized.

Conclusion and Future Directions

The recent renewed emphasis on the Warburg concept suggests that energy metabolism in tumor cells is driven by the increased capacity for aerobic glycolysis, shifting ATP supply away from mitochondrial pathways to meet the energy need for rapid proliferation and effective synthesis of the necessary building blocks. In accordance with this model, multiple glycolytic enzymes tend to be overex-

pressed, and evidence suggests that this may be accompanied by a suppression of mitochondrial metabolic pathways. Figure 1 illustrates the major sites of interaction between signaling processes and pathways of energy metabolism discussed in this review. However, there are several caveats to this widely accepted model.

First, despite the upregulation of multiple glycolytic enzymes in different tumors, it is not clear how much the tumor cell actually can depend on glycolysis for energy supply when needed. In normal tissues with active glycolysis (e.g. muscle, brain, erythrocytes), the rate of flux through the glycolytic pathway is controlled at multiple sites. These include several of the early steps: the glucose transporter that mediates glucose uptake in the cell, the hexokinase-mediated formation of glucose-6-phosphate, and its further phosphorylation by phosphofructokinase, which controls the distribution between glycolysis and the pentose phosphate pathway. A further site of control in some cell types occurs at the level of pyruvate kinase, one of the sites of glycolytic ATP synthesis, which is subject to control by stress hormones. The increased capacity of the glycolytic pathway in many tumor cells is associated with a marked overexpression of specific isoforms of several of the enzymes that mediate these rate-controlling steps (e.g., hexokinase II, GLUT1, M2-PK). In addition, the suppression of TIGAR in p53-deficient cells would effectively enhance the phosphofructokinse step and increase fructose-1,6-bisphosphate levels. However, the reported changes also include enzymes that would not normally impose restrictions on the glycolytic flux (e.g. PGM, LDH). These changes would be expected to shift the flux control to different sites, depending on the relative changes in activity associated with the overexpression of these enzymes and the steady state level of the relevant metabolites and regulatory molecules. In fact, overexpression of PK appears to involve mostly the inactive dimeric form of M2-PK (Mazurek et al. 2005) and activation of this enzyme requires the formation of tetramers, in response to accumulation of substantial levels of the glycolytic intermediate fructose-1,6,-bisphosphate. Therefore, significant flux control at the PK site is expected for tumor cells expressing this isoform of pyruvate kinase, with the relative contribution of other steps being dependent on their effect on intermediate level that can relieve the inhibition of pyruvate kinase activity. A result of these events would be the accumulation of glycolytic intermediates to serve as precursors for synthetic processes (Mazurek et al. 2005). This by itself implies that the distribution of flux through the glycolytic pathway is altered when compared with nontransformed cells. There have been, to our knowledge, no detailed flux control studies of the glycolytic pathway in tumor cells. Moreover, as pointed out by Moreno-Sanchez et al. (2007), different types of tumor do not uniformly express the same complement of alterations in the pattern of glycolytic enzymes. Therefore, the control features of energy metabolism and its ability to respond to hypoxic conditions or other metabolic stress would have to be assessed for individual cell types.

Second, even in tumor cells that have high rates of aerobic glycolysis, the metabolic interactions with the mitochondrial metabolic pathways are critical elements in the overall energetic balance. Some restrictions may be imposed by a more limited capacity of the processes that mediate the metabolic connections

between cytosol and mitochondria, as has been suggested for the malate-aspartate hydrogen shuttle capacity that would normally provide for the oxidation of cytosolic NADH through the mitochondrial electron transport chain. However, the rate of these processes is probably highly dependent on the functional state of the cell and is determined predominantly by the steady state concentrations of the relevant metabolites reflecting the activity of competing processes, such as the activity of LDH. Also, pyruvate oxidation through mitochondrial pathways may be restricted by the limitations imposed on PDH by the activation of the PDH kinase-1, which would suggest there is scope for a dynamic metabolic state. Even with an excess capacity for glycolysis, the cellular energy supply will remain at least in part dependent on mitochondrial metabolism. Many tumor cells survive well on a mitochondrial substrate, such as glutamine.

A more prominent basis for the suppression of mitochondrial metabolism may be the demand for metabolic intermediates in a rapidly growing tumor cell. TCA cycle metabolism fulfills many roles in addition to the supply of electrons to the respiratory chain. A rapidly growing cell has a constant need for a metabolic intermediates, including amino acids and lipid precursors for rapid cell proliferation (Mazurek et al. 2002; Swinnen et al. 2006) many of which derive from TCA cycle intermediates, such as citrate, α-ketoglutarate, and oxaloacetate. The demand for these intermediates may interfere with maximizing mitochondrial energy metabolism. As it is, despite the availability of a great deal of information on the protein constituents that differentiate tumor cells from normal tissues, the relationship between glycolytic flux and mitochondrial energy metabolism in tumor cells remain largely unknown in quantitative terms. A more systematic study of the regulation of TCA cycle activity in the context of metabolic requirements of the cell would be required to resolve these issues, which are difficult to evaluate in terms of protein expression levels alone.

Such studies would also be required to obtain a better understanding of the regulatory processes that determine the flux distribution across the mitochondrial membrane. There is evidence that an increase in mitochondrial metabolic capacity may slow down tumor cell growth and inhibit the tumorigenic potential. This would indicate that the suppression of mitochondrial energy metabolism facilitates tumor cell metabolism. Recent studies suggest that AMPK may be involved in the mechanism responsible for mediating this interaction, possibly in a p53-dependent manner (Wu et al. 2007; Buzzai et al. 2007). This would link the interaction between these two pathways of energy supply through the central energy sensor pathway of the cell and provide another mechanism to integrate the energy demand directly to the p53 stress sensor mechanism. In p53-deficient tumor cells this mechanism would be disrupted. Buzzai et al. provide evidence that the glycolytic control and autophagy can be reestablished by reactivation of AMPK by metformin (admittedly at exceedingly high concentrations that effectively shut down mitochondrial respiration) (Buzzai et al. 2007).

To what extent can these pathways contribute to the suppression of apoptosis in tumor cells? It is now apparent that many of the signaling pathways that control the response to apoptosis, such as p53 or Akt, also influence the metabolic responses of

the tumor cells, and, conversely, signaling pathways that are viewed primarily as metabolic regulatory signals, such as AMPK, affect the cell death response, either through apoptosis or through their impact on autophagic cell death. However, it remains unclear how these two processes are integrated, i.e., how the anomalous metabolic state of the tumor cell is quantitatively translated into a suppression of apoptosis. One view is that a deregulation of apoptotic pathways is simply effective as a survival mechanism for tumor cells that are often exposed to stressful conditions of nutrient and oxygen depletion and oxidative stress: disconnecting the regulation of apoptosis from the metabolic stress response itself provides protection. However, a more direct relationship is not out of bounds. The most immediate example of this remains the mitochondrial binding of hexokinase II, which has a recognized dual role as a metabolic enzyme with direct antiapoptotic effects. What has not been adequately addressed is whether the metabolic consequences of mitochondrial binding of hexokinase II directly contribute to the suppression of the mitochondrial pathway of apoptosis. The classical view of the metabolic consequence of hexokinase II binding to VDAC is that the preferential access of the enzyme to mitochondrial ATP synthesis would help spike the glycolytic pathway (Pedersen et al. 2002). However, this would also stimulate coupled mitochondrial electron transport and depend on the supply of electrons to the mitochondria. Whether the suppression of apoptosis by hexokinase II binding is functionally related to this or other metabolic effects has not been explored.

The recent explosion of interest in the metabolic context of tumor function has as its main objective the development of novel therapies that aim to suppress the tumor cell's growth potential and increase its sensitivity to chemotherapeutic agents. In fact, the recent study by Buzzai et al. (2007) was inspired by epidemiological data that the use of the antidiabetic drug metformin was associated with a lower incidence of cancer, suggesting that the reactivation of AMPK may help suppress tumor growth or survival. In studies from our laboratory, the susceptibility to chemotherapeutic agents, such as cisplatin, was markedly enhanced in tumor cells by detaching hexokinase II from the mitochondria. These few examples suggest that a better understanding of the relationship between energy metabolism and cell death has great promise.

References

Assaily, W. and S. Benchimol, *Differential utilization of two ATP-generating pathways is regulated by p53*. Cancer Cell, 2006. **10**(1): 4–6.

Bernardi, R., et al., *PML inhibits HIF-1alpha translation and neoangiogenesis through repression of mTOR*. Nature, 2006. **442**(7104): 779–85.

Bhaskar, P.T. and N. Hay, *The two TORCs and Akt*. Dev Cell, 2007. **12**(4): 487–502.

Bijur, G.N. and R.S. Jope, *Glycogen synthase kinase-3 beta is highly activated in nuclei and mitochondria*. Neuroreport, 2003. **14**(18): 2415–9.

Bonnet, S., et al., *A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth*. Cancer Cell, 2007. **11**(1): 37–51.

Boren, J., et al., *Gleevec (STI571) influences metabolic enzyme activities and glucose carbon flow toward nucleic acid and fatty acid synthesis in myeloid tumor cells*. J Biol Chem, 2001. **276** (41): 37747–53.

Brdiczka, D., et al., *The molecular structure of mitochondrial contact sites. Their role in regulation of energy metabolism and permeability transition*. Biofactors, 1998. **8**(3-4): 235–42.

Burgering, B.M. and G.J. Kops, *Cell cycle and death control: long live Forkheads*. Trends Biochem Sci, 2002. **27**(7): 352–60.

Buzzai, M., et al., *Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth*. Cancer Res, 2007. **67**(14): 6745–52.

Calissano, P., et al., *Synthesis and content of a DNA-binding protein with lactic dehydrogenase activity are reduced by nerve growth factor in the neoplastic cell line PC12*. Exp Cell Res, 1985. **161**(1): 117–29.

Cantley, L.C. and B.G. Neel, *New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3–kinase/AKT pathway*. Proc Natl Acad Sci USA, 1999. **96**(8): 4240–5.

Carling, D., *The AMP-activated protein kinase cascade-A unifying system for energy control*. Trends Biochem Sci, 2004. **29**(1): 18–24.

Casey, J.R., *Why bicarbonate*? Biochem Cell Biol, 2006. **84**(6): 930–9.

Cattaneo, A., et al., *Nuclear localization of a lactic dehydrogenase with single-stranded DNA-binding properties*. Exp Cell Res, 1985. **161**(1): 130–40.

Chesney, J., *6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and tumor cell glycolysis*. Curr Opin Clin Nutr Metab Care, 2006. **9**(5): 535–9.

Christofk, H.R. et al., *Pyruvate kinase M2 is a phosphotyrosine-binding protein*. Nature, 2008a, **452**: 181–6.

Christofk, H.R. et al., *The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumor growth*. Nature, 2008b. **452**: 230–3.

Choo, A.Y. and J. Blenis, *TORgeting oncogene addiction for cancer therapy*. Cancer Cell, 2006. **9** (2): 77–9.

Discher, D.J., et al., *Hypoxia regulates beta-enolase and pyruvate kinase-M promoters by modulating Sp1/Sp3 binding to a conserved GC element*. J Biol Chem, 1998. **273**(40): 26087–93.

Dombrauckas, J.D., B.D. Santarsiero, and A.D. Mesecar, *Structural basis for tumor pyruvate kinase M2 allosteric regulation and catalysis*. Biochemistry, 2005. **44**(27): 9417–29.

Edinger, A.L. and C.B. Thompson, *Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake*. Mol Biol Cell, 2002. **13**(7): 2276–88.

Fang, J., et al., *The H+-linked monocarboxylate transporter (MCT1/SLC16A1): a potential therapeutic target for high–risk neuroblastoma*. Mol Pharmacol, 2006. **70**(6): 2108–15.

Fantin, V.R., J. St-Pierre, and P. Leder, *Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance*. Cancer Cell, 2006. **9** (6): 425–34.

Fell, D., *Understanding the Control of Metabolism*. 1997: Portland Press, London.

Fell, D.A., *Increasing the flux in metabolic pathways: A metabolic control analysis perspective*. Biotechnol Bioeng, 1998. **58**(2-3): 121–4.

Fischer, K., et al., *Inhibitory effect of tumor cell-derived lactic acid on human T cells*. Blood, 2007. **109**(9): 3812–9.

Forde, J.E. and T.C. Dale, *Glycogen synthase kinase 3: A key regulator of cellular fate*. Cell Mol Life Sci, 2007. **64**(15): 1930–44.

Frey, T.G. and C.A. Mannella, *The internal structure of mitochondria*. Trends Biochem Sci, 2000. **25**(7): 319–24.

Gatenby, R.A. and R.J. Gillies, *Why do cancers have high aerobic glycolysis*? Nat Rev Cancer, 2004. **4**(11): 891–9.

Granville, D.J. and R.A. Gottlieb, *The mitochondrial voltage-dependent anion channel (VDAC) as a therapeutic target for initiating cell death*. Curr Med Chem, 2003. **10**(16): 1527–33.

Hardie, D.G., *The AMP-activated protein kinase pathway-New players upstream and downstream*. J Cell Sci, 2004. **117**(Pt 23): 5479–87.

Hardie, D.G., *New roles for the LKB1?AMPK pathway*. Curr Opin Cell Biol, 2005. **17**(2): 167–73.

Hardt, P.D., et al., *Faecal tumour M2 pyruvate kinase: A new, sensitive screening tool for colorectal cancer*. Br J Cancer, 2004. **91**(5): 980–4.

Hay, N., *The Akt-mTOR tango and its relevance to cancer*. Cancer Cell, 2005. **8**(3): 179–83.

Imai, K., et al., *LKB1, an upstream AMPK kinase, regulates glucose and lipid metabolism in cultured liver and muscle cells*. Biochem Biophys Res Commun, 2006. **351**(3): 595–601.

Inoki, K., et al., *TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling*. Nat Cell Biol, 2002. **4**(9): 648–57.

Inoki, K., T. Zhu, and K.L. Guan, *TSC2 mediates cellular energy response to control cell growth and survival*. Cell, 2003. **115**(5): 577–90.

Inoki, K., et al., *TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth*. Cell, 2006. **126**(5): 955–68.

Jones, R.G., et al., *AMP-activated protein kinase induces a p53-dependent metabolic checkpoint*. Mol Cell, 2005. **18**(3): 283–93.

Kacser, H. and J.A. Burns, *The control of flux*. Symp Soc Exp Biol, 1973. **27**: 65–104.

Kato, K., et al., *Critical roles of AMP-activated protein kinase in constitutive tolerance of cancer cells to nutrient deprivation and tumor formation*. Oncogene, 2002. **21**(39): 6082–90.

Kim, A.H., et al., *Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1*. Mol Cell Biol, 2001. **21**(3): 893-901.

Kim, J.W. and C.V. Dang, *Multifaceted roles of glycolytic enzymes*. Trends Biochem Sci, 2005. **30** (3): 142–50.

Kim, J.W. and C.V. Dang, *Cancer's molecular sweet tooth and the Warburg effect*. Cancer Res, 2006. **66**(18): 8927–30.

Kim, J.W., et al., *HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia*. Cell Metab, 2006. **3**(3): 177–85.

King, A., M.A. Selak, and E. Gottlieb, *Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer*. Oncogene, 2006. **25**(34): 4675–82.

Kruse, J.P. and W. Gu, *p53 aerobics: The major tumor suppressor fuels your workout*. Cell Metab, 2006. **4**(1): 1–3.

Kumar, Y., et al., *Tumour M2-pyruvate kinase: A gastrointestinal cancer marker*. Eur J Gastroenterol Hepatol, 2007. **19**(3): 265–76.

Li, Y., et al., *TSC2: Filling the GAP in the mTOR signaling pathway*. Trends Biochem Sci, 2004. **29**(1): 32–8.

Luo, Z., et al., *AMPK, the metabolic syndrome and cancer*. Trends Pharmacol Sci, 2005. **26**(2): 69–76.

Ma, W., et al., *A pivotal role for p53: Balancing aerobic respiration and glycolysis*. J Bioenerg Biomembr, 2007.

MacKenzie, E.D., et al., *Cell-permeating alpha-ketoglutarate derivatives alleviate pseudohypoxia in succinate dehydrogenase-deficient cells*. Mol Cell Biol, 2007. **27**(9): 3282–9.

Majewski, N., et al., *Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak*. Mol Cell, 2004. **16**(5): 819–30.

Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: Navigating downstream*. Cell, 2007. **129** (7): 1261–74.

Marchetti, P., et al., *Mitochondrial permeability transition is a central coordinating event of apoptosis*. Journal of Experimental Medicine, 1996. **184**(3): 1155–60.

Marshall, S., *Role of insulin, adipocyte hormones, and nutrient-sensing pathways in regulating fuel metabolism and energy homeostasis: A nutritional perspective of diabetes, obesity, and cancer*. Sci STKE, 2006. **2006**(346): re7.

Mathupala, S.P., C. Heese, and P.L. Pedersen, *Glucose catabolism in cancer cells. The type II hexokinase promoter contains functionally active response elements for the tumor suppressor p53*. J Biol Chem, 1997. **272**(36): 22776–80.

Mathupala, S.P., Y.H. Ko, and P.L. Pedersen, *Hexokinase II: Cancer' s double–edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria*. Oncogene, 2006. **25**(34): 4777–86.

Matoba, S., et al., *p53 regulates mitochondrial respiration*. Science, 2006. **312**(5780): 1650–3.

Mazurek, S., C.B. Boschek, and E. Eigenbrodt, *The role of phosphometabolites in cell proliferation, energy metabolism, and tumor therapy*. J Bioenerg Biomembr, 1997. **29**(4): 315–30.

Mazurek, S., et al., *Pyruvate kinase type M2: A crossroad in the tumor metabolome*. Br J Nutr, 2002. **87**(Suppl 1): S23–9.

Mazurek, S., et al., *Pyruvate kinase type M2 and its role in tumor growth and spreading*. Semin Cancer Biol, 2005. **15**(4): 300–8.

Minchenko, A., et al., *Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect*. J Biol Chem, 2002. **277**(8): 6183–7.

Modur, V., et al., *FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer*. J Biol Chem, 2002. **277**(49): 47928–37.

Moll, U.M., N. Marchenko, and X.K. Zhang, *p53 and Nur77/TR3-Transcription factors that directly target mitochondria for cell death induction*. Oncogene, 2006. **25**(34): 4725–43.

Moreno-Sanchez, R., et al., *Energy metabolism in tumor cells*. Febs J, 2007. **274**(6): 1393–418.

Papandreou, I., et al., *HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption*. Cell Metab, 2006. **3**(3): 187–97.

Pastorino, J.G. and J.B. Hoek, *Hexokinase II: The integration of energy metabolism and control of apoptosis*. Curr Med Chem, 2003. **10**(16): 1535–51.

Pastorino, J.G. and J.B. Hoek, *Regulation of hexokinase binding to VDAC*. J Bioenergetics Biomembranes, 2008. **40**: 171–82.

Pastorino, J.G., N. Shulga, and J.B. Hoek, *Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis*. J Biol Chem, 2002. **277**(9): 7610–8.

Pastorino, J.G., J.B. Hoek, and N. Shulga, *Activation of glycogen synthase kinase 3beta disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity*. Cancer Res, 2005. **65**(22): 10545–54.

Pedersen, P.L., et al., *Mitochondrial bound type II hexokinase: A key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention*. Biochim Biophys Acta, 2002. **1555**(1-3): 14–20.

Pelicano, H., et al., *Glycolysis inhibition for anticancer treatment*. Oncogene, 2006. **25**(34): 4633–46.

Plas, D.R. and C.B. Thompson, *Akt-dependent transformation: There is more to growth than just surviving*. Oncogene, 2005. **24**(50): 7435–42.

Provent, P., et al., *Serial in vivo spectroscopic nuclear magnetic resonance imaging of lactate and extracellular pH in rat gliomas shows redistribution of protons away from sites of glycolysis*. Cancer Res, 2007. **67**(16): 7638–45.

Reiling, J.H. and E. Hafen, *The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila*. Genes Dev, 2004. **18**(23): 2879–92.

Roe, J.S., et al., *p53 stabilization and transactivation by a von Hippel-Lindau protein*. Mol Cell, 2006. **22**(3): 395–405.

Rutter, G.A., G. Da Silva Xavier, and I. Leclerc, *Roles of 5 -AMP-activated protein kinase (AMPK) in mammalian glucose homoeostasis*. Biochem J, 2003. **375**(Pt 1): 1–16.

Schwarzer, R., et al., *REDD1 integrates hypoxia-mediated survival signaling downstream of phosphatidylinositol 3-kinase*. Oncogene, 2005. **24**(7): 1138–49.

Semenza, G.L., *HIF-1 mediates the Warburg effect in clear cell renal carcinoma*. J Bioenerg Biomembr, 2007.

Shaw, R.J., *Glucose metabolism and cancer*. Curr Opin Cell Biol, 2006. **18**(6): 598–608.

Shim, H., et al., *c-Myc transactivation of LDH-A: Implications for tumor metabolism and growth*. Proc Natl Acad Sci USA, 1997. **94**(13): 6658–63.

Shimizu, S., M. Narita, and Y. Tsujimoto, *Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC*. Nature, 1999. **399**(6735): 483–7.

Stetak, A., et al., *Nuclear translocation of the tumor marker pyruvate kinase M2 induces programmed cell death*. Cancer Res, 2007. **67**(4): 1602–8.

Sui, D. and J.E. Wilson, *Structural determinants for the intracellular localization of the isozymes of mammalian hexokinase: Intracellular localization of fusion constructs incorporating structural elements from the hexokinase isozymes and the green fluorescent protein*. Arch Biochem Biophys, 1997. **345**(1): 111–25.

Sun, X., et al., *Overexpression of von Hippel-Lindau tumor suppressor protein and antisense HIF-1alpha eradicates gliomas*. Cancer Gene Ther, 2006. **13**(4): 428–35.

Sunters, A., et al., *FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines*. J Biol Chem, 2003. **278**(50): 49795–805.

Swinnen, J.V., K. Brusselmans, and G. Verhoeven, *Increased lipogenesis in cancer cells: New players, novel targets*. Curr Opin Clin Nutr Metab Care, 2006. **9**(4): 358–65.

Tomita, K., et al., *AICAR, an AMPK activator, has protective effects on alcohol-induced fatty liver in rats*. Alcohol Clin Exp Res, 2005. **29**(12 Suppl): 240S–5S.

Vander Heiden, M.G., et al., *Growth factors can influence cell growth and survival through effects on glucose metabolism*. Mol Cell Biol, 2001. **21**(17): 5899–912.

Vyssokikh, M.Y., et al., *Bax releases cytochrome c preferentially from a complex between porin and adenine nucleotide translocator. Hexokinase activity suppresses this effect*. Mol Biol Rep, 2002. **29**(1-2): 93–6.

Warburg, O., *Metabolism of Tumors*. 1930: Arnold Constable, London.

Watcharasit, P., et al., *Glycogen synthase kinase-3beta (GSK3beta) binds to and promotes the actions of p53*. J Biol Chem, 2003. **278**(49): 48872–9.

Weinhouse, S., *Glycolysis, respiration, and anomalous gene expression in experimental hepatomas: G.H.A. Clowes memorial lecture*. Cancer Res, 1972. **32**(10): 2007–16.

Williams, K.R., S. Reddigari, and G.L. Patel, *Identification of a nucleic acid helix-destabilizing protein from rat liver as lactate dehydrogenase-5*. Proc Natl Acad Sci USA, 1985. **82**(16): 5260–4.

Wilson, J.E., *Isozymes of mammalian hexokinase: Structure, subcellular localization and metabolic function*. J Exp Biol, 2003. **206**(Pt 12): 2049–57.

Wilson, J.E., *Hexokinases*. Rev Physiol Biochem Pharmacol, 1995. **126**: 65–198.

Woods, A., et al., *LKB1 is the upstream kinase in the AMP-activated protein kinase cascade*. Curr Biol, 2003. **13**(22): 2004–8.

Wu, M., et al., *Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells*. Am J Physiol Cell Physiol, 2007. **292**(1): C125–C36.

Xie, G.C. and J.E. Wilson, *Rat brain hexokinase: The hydrophobic N–terminus of the mitochondrially bound enzyme is inserted in the lipid bilayer*. Arch Biochem Biophys, 1988. **267**(2): 803–10.

Yu, J., et al., *PUMA induces the rapid apoptosis of colorectal cancer cells*. Mol Cell, 2001. **7**(3): 673–82.

Zhang, H., et al., *HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity*. Cancer Cell, 2007. **11**(5): 407–20.

Zhang, L., et al., *Role of BAX in the apoptotic response to anticancer agents*. Science, 2000, **290**: 989–92.

Zhu, W., et al., *Regulation of FOXO3a by brain-derived neurotrophic factor in differentiated human SH-SY5Y neuroblastoma cells*. Brain Res Mol Brain Res, 2004. **126**(1): 45–56.

Zuurbier, C.J., O. Eerbeek, and A.J. Meijer, *Ischemic preconditioning, insulin, and morphine all cause hexokinase redistribution*. Am J Physiol Heart Circ Physiol, 2005. **289**(1): H496–H9.

Energy Generating Pathways and the Tumor Suppressor p53

Chad A. Corcoran, Ying Huang, and M. Saeed Sheikh

Introduction

Energy generating metabolic pathways are essential for sustaining life in all cells by producing ATP, the currency for cellular transactions. Without ATP production, the driving force behind countless energetically unfavorable enzymatic reactions would not be present and life in its current form would not exist. Thus, there are compelling reasons to understand how the chemical energy harnessed within the covalent bonds of ATP is both produced and transferred. In this regard, the study of ATP synthesis typically begins with the breakdown of glucose through the anaerobic, or oxygen-independent reactions of glycolysis and follows a path through the citric acid cycle and the aerobic, oxygen-dependent process of oxidative phosphorylation/aerobic respiration (Fig. 1). Of the multiple enzymes involved in these processes, only those of the citric acid cycle and oxidative phosphorylation are localized within mitochondria. However, because the cytosolic process of glycolysis is essential for producing pyruvate, the substrate for the citric acid cycle, a complete understanding of all processes is necessary to understand how cytosolic glucose breakdown eventually leads to the substantial amounts of ATP generated within mitochondrial compartments.

In context to cancer, the regulation of energy generating pathways, like so many other processes, is markedly altered. Now commonly referred to as the Warburg effect, the rate of glycolysis in cancer cells significantly increases with a concomitant decrease in aerobic respiration (Warburg 1956). The reason for the shift could be the onset of hypoxic conditions within the growing tumor, thus necessitating the production of anaerobic energy to sustain aberrant growth. Though the mechanisms leading to increased glycolysis even in the presence of oxygen remain to be fully elucidated, the application of this decades-old observation can be seen today as positron emission tomography (PET) that takes advantage of the enhanced glucose uptake and glycolytic rate to identify both primary and metastatic tumors.

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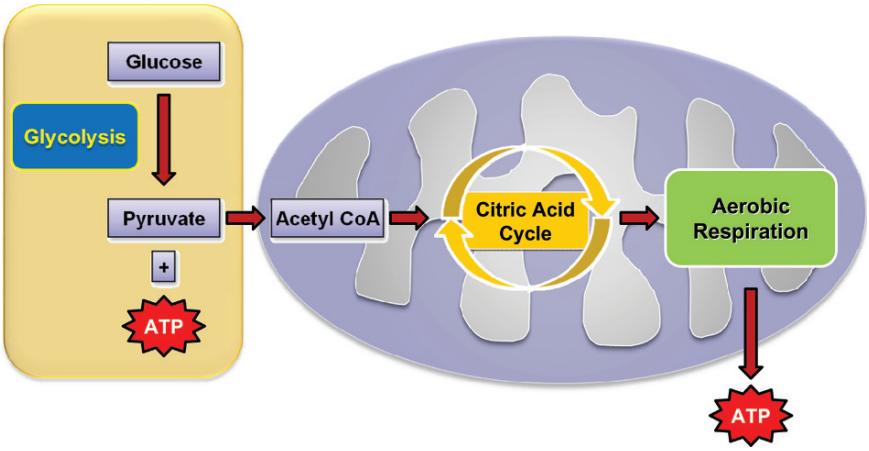

Fig. 1 Compartmentalization of glycolysis, the citric acid cycle, and aerobic respiration. The conversion of glucose to pyruvate and the anaerobic generation of ATP by glycolysis are carried out entirely in the cytosol. The generated pyruvate is then imported into the mitochondria, where it is converted to acetyl coenzyme A (acetyl CoA) and metabolized in the citric acid cycle. Enzymes of the aerobic respiration pathway are localized to the inner mitochondrial membrane, where large amounts of ATP are generated and pool in the mitochondrial matrix. ATP is later transported out of the mitochondria into the cytosol, where it can be utilized in energy-dependent processes

Thus, although the relevancy of increased glycolysis and decreased aerobic respiration in human cancer has been realized in basic concept and translated to clinical application, a more complete understanding of the mechanisms that lead to changes in these processes will be a linchpin for developing therapeutic strategies to target these vital metabolic pathways. In this regard, the tumor suppressor p53 is emerging as an important regulator of both glycolytic and aerobic respiratory pathways. The intimate link between p53 and cancer development and progression is well-established. It is capable of sensing multiple stresses, including hypoxia, and responding by regulating the expression and function of specific downstream effector proteins (Prives and Hall 1999; Vogelstein, Lane and Levine 2000; Oren 2003). As a transcription factor, p53 has long been known to initiate signaling cascades important to cell growth inhibition and apoptosis. However, the discovery that p53 also mediates its effects through life sustaining metabolic pathways gives yet another dimension to the function of this tumor suppressor protein. Furthermore, multiple lines of evidence are solidifying a role for p53 in negative growth control via its ability to participate in the apoptotic signaling cascades directly at the mitochondrial level (Perfettini, Kroemer and Kroemer 2004; Erster and Moll 2005). Therefore, the idea that p53 regulates energy generating metabolic pathways both transcriptionally and via its direct role at the mitochondria appears all the more fascinating. This chapter aims to focus on the current state of knowledge on the molecular basis for the interplay between p53 and the proteins of glycolysis and aerobic respiration.

Regulation of Proteins Involved in Glycolysis by p53

Proteins Directly Involved in Glycolysis

Type II Hexokinase

The early evidence indicating p53 might be involved in the modulation of glycolysis first appeared in studies performed by Pedersen and colleagues (Mathupala et al. 1997). The authors discovered that p53 was involved in upregulating the expression of type II hexokinase, the enzyme implicated in the first step of glycolysis, converting glucose to glucose-6-phosphate (G6P; Fig. 2; Mathupala et al. 1997). Further studies indicated this upregulation via p53 to be direct by uncovering the presence of functional p53 response elements in the hexokinase II promoter (Mathupala et al. 1997). These findings appeared to be somewhat contradictory given that p53 is a potent tumor suppressor and hexokinase II contributes to the increased glycolysis seen as a hallmark of malignancy. Because hexokinase II is overexpressed in various human malignancies (Smith 2000; Tian et al. 2005), upregulation of hexokinase II by p53 would suggest a further increase in glycolytic

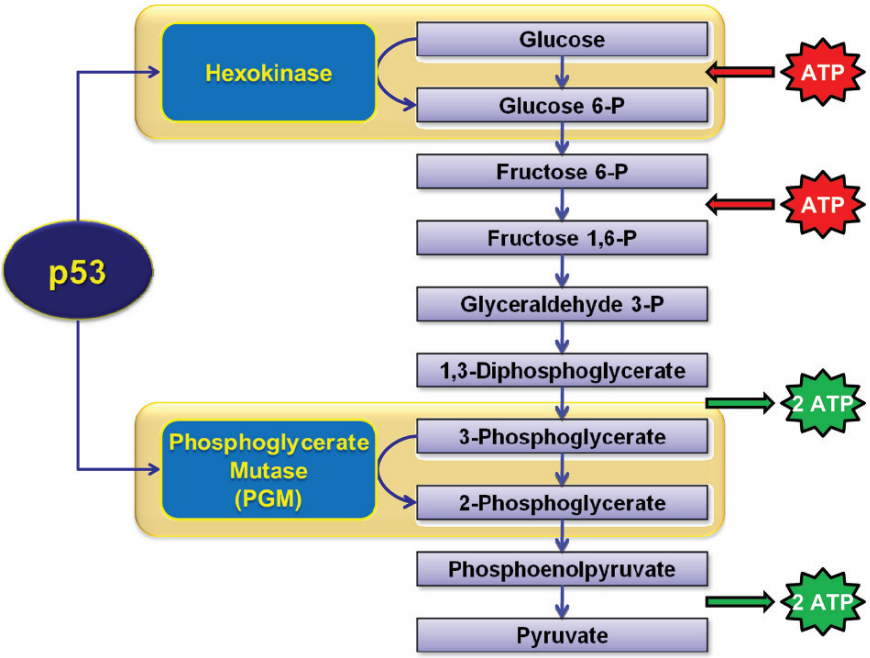

Fig. 2 The regulation of glycolysis by p53. Glycolysis converts glucose to pyruvate through a series of reactions, each of which is performed by a different enzyme. p53 appears to regulate the expression of type II hexokinase and phosphoglycerate mutase

activity. Interestingly, other examples of p53-mediated upregulation of potential anti-apoptotic/growth promoting proteins are well documented. Upregulation of both the epidermal growth factor receptor (EGFR) as well as heparin-binding epidermal growth factor (HB-EGF) by p53 have both been demonstrated (Ludes-Meyers et al. 1996; Fang et al. 2001). Furthermore, p53 transcriptional activation of HB-EGF has been shown to activate the PI3K/Akt/MAPK/Ras signaling pathway, that similar to glycolysis, promotes aberrant tumor cell growth and is deregulated in human cancer (Fang et al. 2001). It has been proposed that p53-mediated activation of PI3K/Akt/MAPK/Ras signaling serves as a compensatory response to protect cells against stress; however, whether enhanced glycolysis due to hexokinase II upregulation functions similarly remains to be determined. Alternatively, whether p53 upregulates hexokinase II in order to abate its own potent growth-inhibitory and pro-apoptotic response is another issue that requires future examination. This too would not be without precedence as it has been established that p53 upregulates several proteins involved in inhibiting its tumor suppressor function and/or targeting it for degradation, including for example, the E3 ubiquitin ligases MDM2, Pirh2, and COP1 (Corcoran et al. 2004).

What makes p53-mediated hexokinase II upregulation even more intriguing is the starkly divergent roles each protein plays at the mitochondria. Although it is a soluble cytosolic protein, a vast majority of hexokinase II is localized to the outer mitochondrial membrane bound to the voltage dependent anion channel (VDAC; Fig. 3; Arora and Pedersen 1988; Nakashima et al. 1986; Forte et al. 1987). At the mitochondria hexokinase II is thought to enhance its enzymatic activities by

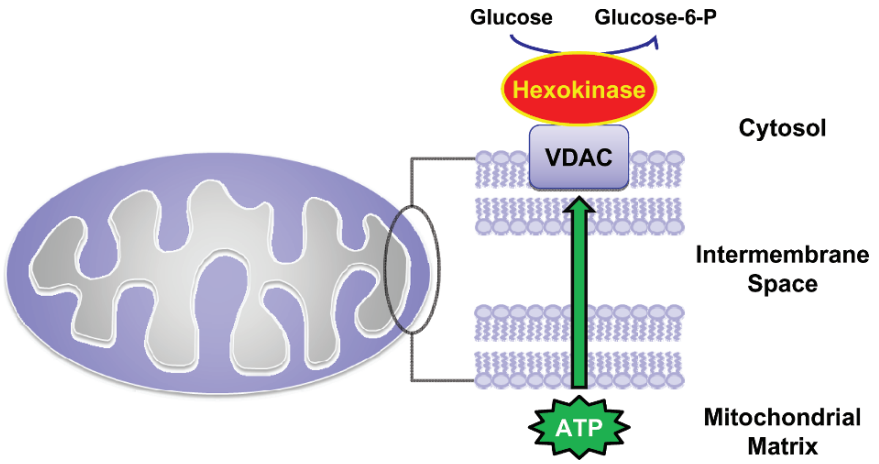

Fig. 3 Hexokinase is localized to the mitochondria. Although glycolysis takes place exclusively in the cytosol, it is well-established that hexokinase catalyzes the conversion of glucose to glucose-6-phosphate at the outer mitochondrial membrane (Parry and Pedersen 1983; Arora and Pedersen 1988). Through an interaction with the voltage-dependent anion channel (VDAC; Arora and Pedersen 1988; Nakashima et al. 1986; Forte et al. 1987), hexokinase is thought to have preferential access to mitochondrial ATP pools, thereby enhancing its catalytic potential

preferentially utilizing aerobic respiration-derived ATP pools to carry out the phosphorylation of glucose to G6P (Parry and Pedersen 1983; Arora and Pedersen 1988). In addition to enhancing its enzymatic function, the mitochondrial localization of hexokinase II appears to inhibit the release of cytochrome c and apoptosis perhaps by antagonizing pro-apoptotic members of the Bcl-2 family, including Bax, Bak, and Bid (Fig. 4a; Bryson et al. 2002; Pastorino et al. 2002; Majewski et al. 2004a, b). p53, by contrast, has recently been implicated in facilitating mitochondria-based apoptotic signaling through its direct interactions with Bax, Bak, Bcl-2, and Bcl-X_L at the outer mitochondrial membrane (Fig. 4b; Mihara et al. 2003; Chipuk et al. 2004; Leu et al. 2004; Perfettini et al. 2004; Erster and Moll 2005). Hence, the functional significance of p53-mediated hexokinase II expression appears to be quite complex and may extend far beyond hexokinase II's role in the initial step of glycolysis.

Although at the present time they appear to be contradictory in nature, these initial studies have suggested hexokinase II, a key glycolytic enzyme, is a p53-regulated protein that appears to play a role in converging the seemingly independent processes of apoptosis and glycolysis at the mitochondria. The critical questions that now warrant further investigations include (i) why p53 potentiates glycolysis and (ii) what additional roles hexokinase II plays in modulating the apoptotic signaling events.

Phosphoglycerate Mutase (PGM)

Recently, Kondoh et al. (2005) reported that in mouse embryo fibroblasts (MEFs) p53 was capable of downregulating phosphoglycerate mutase (PGM) expression and enzymatic activity. PGM converts 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) downstream of hexokinase II in the glycolytic pathway (Fig. 2). Since PGM activity has been demonstrated to be markedly enhanced in several cancer types, including breast and colon (Durany et al. 1997a, b, 2000), downregulation of PGM would be more consistent with the accepted notion that p53 would inhibit cancer cell growth and proliferation by negatively regulating glycolytic productivity. However, a conflicting report utilizing rat cardiomyocytes has shown PGM to be a direct p53 transcriptional target (Ruiz-Lozano et al. 1999) thus, opposing the results obtained from studies done in MEFs. While the same glycolytic enzymes exist within virtually all eukaryotic organisms, the pathways that regulate their expression are poorly understood and could very well differ from species to species. Thus, further studies utilizing human cancer cell types would likely provide additional insight into p53-mediated PGM regulation in human malignancy. Nonetheless, studies on the regulation of PGM expression by p53 have again shown the potential for p53 to modulate the glycolytic pathway and has raised the very fascinating possibility that p53 might control the expression of still more proteins directly involved in glycolysis.

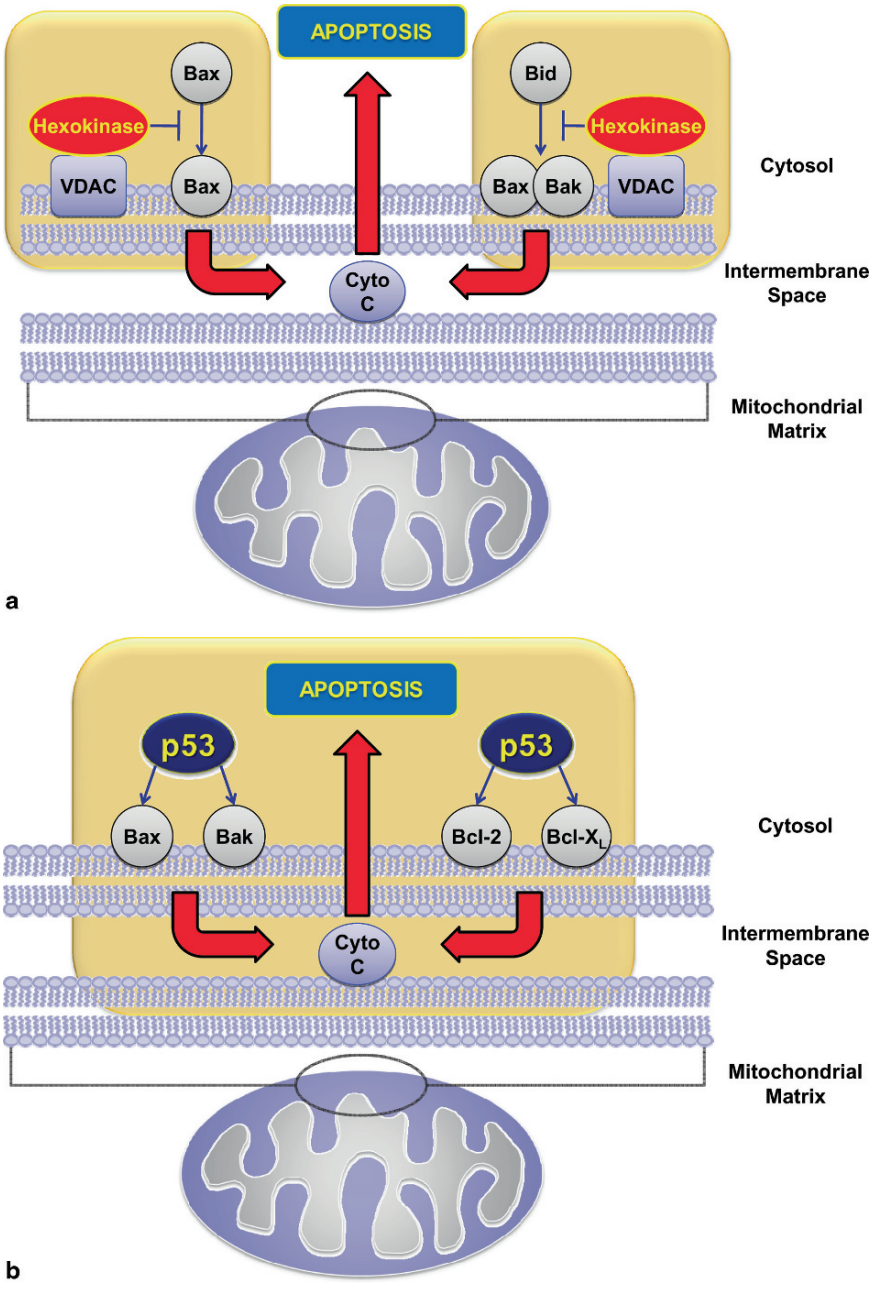

Fig. 4 Mutual interactions among hexokinase, p53, and proteins of the mitochondrial apoptotic pathway. (**a**) Hexokinase inhibition of cytochrome c release and apoptosis. Hexokinase II bound to the mitochondria has been shown to inhibit cytochrome c release and apoptosis by either interfering with the mitochondrial localization of pro-apoptotic protein Bax (left; Pastorino et al. 2002)

Proteins Indirectly Involved in Glycolysis

LKB1 and AMPK

Given the importance of energy generation in both normal cell growth and malignant progression, it would appear logical that mechanisms be set in place by cells to control the coordinated sequence of events carried out by multiple enzymes within the glycolytic pathway. Similarly, the tumor suppressor p53 undergoes extensive posttranslational modifications including phosphorylation, ubiquitination, and sumoylation to ensure that a proper and controlled response to a variety of stresses (Lavin and Gueven 2006). For example, it is well-established that p53 is activated following DNA damage, in part, by ATM (mutated in ataxia-telangiectasia) phosphorylation at multiple serine residues (Lavin and Gueven 2006). It is therefore reasonable to assume that p53 may also be activated by similar means in response to altered metabolism or nutrient availability.

To this end, it was only recently discovered that LKB1 and AMP-activated protein kinase (AMPK) functioned coordinately to sense and regulate the response to changes in glucose levels and the energetic demands of the cell (Shaw et al. 2004; Spicer and Ashworth 2004; Luo et al. 2005; Shaw et al. 2005; Katajisto et al. 2007). LKB1 was found to be an activator of AMPK activity with loss of LKB1 resulting in complete inhibition of AMPK-mediated signaling in LKB1-null MEFs (Shaw et al. 2004; Spicer and Ashworth 2004). Links between LKB1, AMPK and cancer are now just beginning to be established, the most significant of which may be a direct connection to p53. Independent studies have demonstrated LKB1 and AMP kinases each regulate p53 activity through phosphorylation (Imamura et al. 2001; Karuman et al. 2001; Jones et al. 2005; Zeng and Berger 2006; Craig et al. 2007), providing yet another important connection between p53 and the control of glycolysis. Research conducted by Karuman et al. (2001) was the first to show that LKB1 kinase physically associates with p53 and apoptosis induced by LKB1 was partially dependent on the presence of p53. Of significance is the fact that LKB1 translocates to the mitochondria following cell insult, a process that is also mediated by p53 expression. However, whether LKB1 serves to enhance p53 activity at the mitochondria was not evaluated. Moreover, LKB1 knockout mice are hyperglycemic (Shaw et al. 2005), suggesting LKB1 functions to suppress glycolytic flux and that the two proteins may work together to suppress tumorigenesis. This hypothesis is supported by the findings that LKB1$^{+/-}$ mice crossed with mice of p53-null background had increased tumor incidence and shorter life-spans versus mice with deletions of LKB1 or p53 alone (Wei et al. 2005).

Fig. 4 (Continued) or by inhibiting Bid-mediated interactions between pro-apoptotic proteins Bax and Bak (right; Majewski et al. 2004a). (**b**) p53 promotes cytochrome c release and apoptosis. p53 is believed to facilitate transcription-independent apoptosis through direct interactions with either pro-apoptotic proteins, Bax and Bak (left; Chipuk et al. 2004; Leu et al. 2004), or anti-apoptotic proteins, Bcl-2 and Bcl-X_L (right; Mihara et al. 2003)

Similar to LKB1, AMPK phosphorylates p53 at multiple serine residues, leading to its activation and induction of cell-cycle arrest under hypoglycemic conditions (Jones et al. 2005; Craig et al. 2007). Additionally, the β1 and β2 subunits of AMPK appear to be a direct target of p53, thus creating a positive feedback mechanism between the two proteins (Feng et al. 2007). Despite recent progress in understanding the connections between LKB1, AMPK and p53, many question still remain. For instance, although LKB1 and AMPK can activate p53 in hypoglycemia, it is unknown whether either one can elicit the same p53-dependent response under hyperglycemic conditions in malignant cells. Moreover, it is possible that in the hyperglycemic environment of malignant cells LKB1 and AMPK signaling to p53 could be deactivated due to i) the high occurrence of p53 mutations (Royds and Iacopetta 2006; Lim et al. 2007; Petitjean et al. 2007), ii) mutations or deletions of LKB1 in several cancer types (McGarrity and Amos 2006; Sanchez-Cespedes 2007), or iii) deactivation of AMPK which is inhibited by elevated glucose levels (Luo et al. 2005).

TP53-Induced Glycolysis and Apoptosis Regulator (TIGAR)

Still further connections between the tumor suppressor p53 and glucose metabolism has been reported by Vousden and colleagues in a recent study (Bensaad et al. 2006). The authors made the novel discovery that p53-mediated upregulation of TIGAR (TP53-induced glycolysis and apoptosis regulator) resulted in an inhibition of glycolysis. The expression of TIGAR was enhanced in a p53-dependent manner via two p53 consensus binding sites found within the TIGAR promoter as well as by the DNA damage inducing agent, adriamycin. Interestingly, TIGAR bears significant similarity to proteins of the PGM family that are also regulated by p53 (Ruiz-Lozano et al. 1999; Kondoh et al. 2005). More specifically, TIGAR shares homology to the bisphosphatase (BPase) domain of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2). It was demonstrated that like the FBPase domain of PFK-2/FBPase-2, TIGAR expression resulted in decreased fructose-2,6-phosphate (F-2,6-P) levels (Bensaad et al. 2006). Given that F-2,6-P is a potent allosteric activator of PFK-1 (Okar et al. 2001), an early and rate-limiting enzyme in the glycolytic pathway (Fig. 5), TIGAR functions similarly to PFK-2/FBPase-2 by decreasing F-2,6-P levels thereby reducing glycolytic flux.

Earlier studies appear to further add to the relative significance of TIGAR's discovery. For instance, F-2,6-P, in addition to allosterically activating PFK-1, has also been found to enhance expression glucokinase (GK/type IV hexokinase; Wu et al. 2004). Glucokinase is a hexokinase isoform predominantly expressed in hepatocytes and its enhanced expression would be expected to cause increased glycolytic flux. However, TIGAR-mediated decreases in F-2,6-P may prevent such an increase in GK levels, thus providing an additional means by which TIGAR may function to negatively regulate glycolysis. Additional studies are clearly needed to evaluate these possibilities. Further studies are also needed to explore the possibility whether TIGAR has any effect on the expression of other glycolytic enzymes, such as hexokinase II.

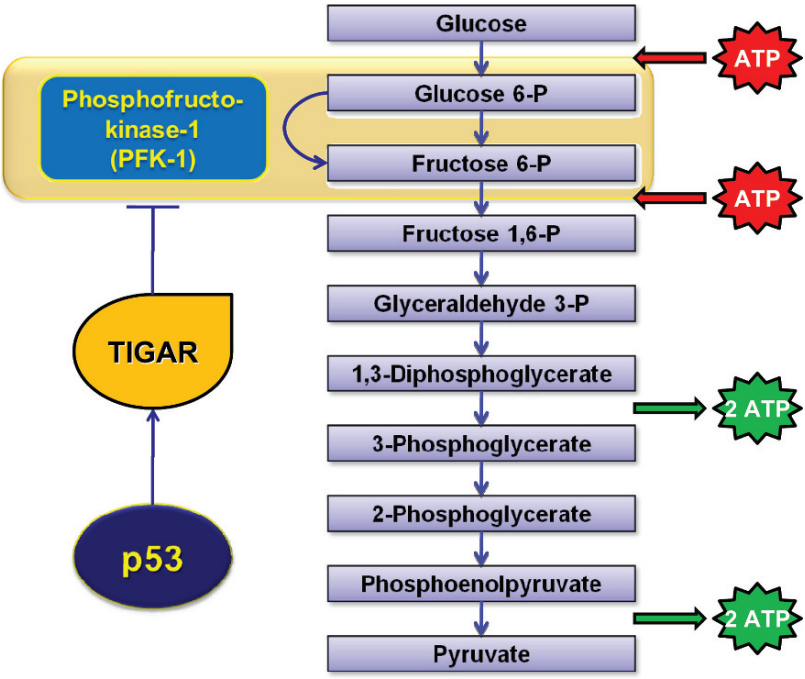

Fig. 5 The indirect regulation of glycolysis by p53. In addition to regulating the expression of hexokinase II and PGM, which are directly involved in glycolysis, p53 also affects glycolysis indirectly, in part through TIGAR (Bensaad et al. 2006). TIGAR negatively affects glycolysis by decreasing the levels of F-2,6-P, which has been shown to allosterically activate PFK-1 (Okar et al. 2001)

Proteins Involved in Glucose Import

GLUT1 and GLUT4

Before glucose can be metabolized, it must first be transported across the plasma membrane into the cytosol. This process is carried out by transmembrane glucose transporters (GLUT), a family of proteins comprising at least thirteen members (Macheda et al. 2005). In addition to hexokinase II overexpression, aberrant expression of GLUTs is considered to be the other critical determinant in enhancing glycolytic rates in malignant tissues (Gatenby and Gillies 2004). By providing glucose that will be metabolized by hexokinase to G6P in the initial step of glycolysis, GLUT proteins essentially provide the fuel for the glycolytic fire. Within the extended GLUT protein family, GLUT1-GLUT4 are the most extensively studied and of these GLUT1, GLUT3, and GLUT4 have each been demonstrated to be aberrantly expressed in a host of different human malignancies, including cancers of the brain, breast, cervix, kidney, lung, liver, ovary, pancreas,

thyroid, and gastrointestinal tract (Gatenby and Gillies 2004; Schwartzenberg-Bar-Yoseph et al. 2004; Macheda et al. 2005). Schwartzenberg-Bar-Yoseph et al. (2004) have shown in vitro binding of p53 to regions of the GLUT1 and GLUT4 promoter providing a direct link between p53 and glucose uptake into the cell. Moreover, mutations in the DNA binding domain of p53 blunted its repressive ability on both promoters (Schwartzenberg-Bar-Yoseph et al. 2004). These findings therefore suggest that in malignancy, where p53 is mutated in approximately 50% of all cancers (Royds and Iacopetta 2006; Lim et al. 2007; Petitjean et al. 2007), GLUT1 and GLUT4 overexpression could, at least in part, result from p53 inactivation. Interestingly, there is at least one report indicating that enhanced glucose concentrations lead to enhanced apoptosis and mitochondrial p53 translocation (Ortega-Camarillo et al. 2006). Hence, both the transcriptional downregulation of GLUT1 and GLUT4 and the p53-mediated initiation of mitochondrial apoptotic signaling cascades could occur through mechanisms designed to trigger p53 activation in response to elevated glucose levels.

Proteins of the Glycolytic Pathway as Potential Therapeutic Targets

Long before the involvement of p53 in regulating glycolytic proteins was reported, strategies for inhibiting glycolysis in human malignancy were already conceived. 2-Deoxyglucose (2DG) is a glucose analog that when phosphorylated to form 2DG-P by hexokinase cannot undergo further modification to progress through the glycolytic pathway (Wick et al. 1955). 2DG has been utilized in the laboratory as an inhibitor of glycolysis for almost 50 years, however, its in vivo effectiveness is potentially limited by the overabundance of glucose which in many cases is due to GLUT overexpression. Nonetheless, numerous studies involving 2DG are currently evaluating its effectiveness as a chemotherapeutic agent (Mohanti et al. 1996; Singh et al. 2005; Kang and Hwang 2006; Pelicano et al. 2006). Even more direct inhibition has been achieved through targeting the specific glycolytic enzymes. For example, the critical role of hexokinase in glycolysis coupled with its overexpression in human cancers has made it an exciting target for therapeutic intervention. Accordingly, lonidamine appears to be a promising agent that functions by inhibiting hexokinase enzymatic function (Floridi et al. 1981a, b; Di Cosimo et al. 2003). Similarly, 3-bromopyruvate is emerging as a clinically relevant drug that also potently inhibits hexokinase activity (Mathupala et al. 2006). Consistent with hexokinase's ability to antagonize mitochondria-based apoptotic signaling (Bryson et al. 2002; Pastorino et al. 2002; Majewski et al. 2004a, b), 3-bromopyruvate also has been show to promote Bax mitochondrial localization and cytochrome c release through dephosphorylation of Bad, likely through hexokinase inhibition (Xu et al. 2005). Thus, 3-bromopyruvate may serve to further enhance the pro-apoptotic response brought on by p53 activation and aid in apoptotic signaling cascades initiated by p53 at the mitochondria. The drugs clotrimazole and bifonazole also target hexokinase albeit by interfering with its subcellular localization to the

mitochondrial membrane (Penso and Beitner 1998). By promoting the displacement of hexokinase from the mitochondria, clotrimazole and bifonazole may also serve to promote permeabilization of the mitochondrial membrane and cytochrome c release to aid in p53-mediated apoptosis. Further studies are however, needed to explore this possibility. A more complete listing of compounds targeting glycolytic enzymes is shown in Table 1. Integration of novel glycolysis-targeted compounds into existing chemotherapeutic strategies will likely have escalating impact on the refinement of future therapeutic approaches. With the recent inception of therapeutic strategies aimed directly at enhancing p53 pro-apoptotic ability (Selivanova and Wiman 2007; Vassilev 2007), the probability that these agents will impact attempts to maximize the tumor suppressor function of p53 will continue to increase.

Table 1 A list of agents known to target the glycolytic pathway

Agent	Target/Mechanism of Action	Reference
2-Deoxyglucose* (2-DG)	Glucose analogue Competes with glucose for hexokinase	Wick, Drury and Morita 1955; Mohanti et al. 1996; Singh et al. 2005; Kang and Hwang 2006
Mannoheptulose	2-DG-P cannot be metabolized by Phosphoglucose isomerase Glucose analogue Inhbits glucokinase activity and glucose uptake	Board et al. 1995
5-Thioglucose	Glucose analogue Inhibits hexokinase activity	Wilson and Chung 1989
Glufosfamide*	Glucose conjugate DNA damage	Veyhl et al. 1998; Seker et al. 2000; Briasoulis et al. 2003; Giaccone et al. 2004
Lonidamine*	Inhibition of mitochondrial-bound hexokinase	Floridi et al. 1981a, b; Di Cosimo et al. 2003
3-Bromopyruvate	Inhibits hexokinase activity	Xu et al. 2005
Clotrimazole	Calmodulin antagonist Interferes with hexokinase localization to the mitochondria Dissociates phosphofructokinase and aldolase from cytoskeleton	Glass-Marmor and Beitner 1997; Penso and Beitner 1998
Bifonazole	Calmodulin antagonist Interferes with hexokinase localization to the mitochondria Dissociates phosphofructokinase and aldolase from cytoskeleton	Glass-Marmor and Beitner 1997; Penso and Beitner 1998
Imatinib*	Inhibits Bcr-Abl tyrosine kinase activity Inhibits hexokinase activity and glucose uptake Enhances citric acid cycle activity	Boren et al. 2001; Gottschalk et al. 2004

*Indicates agents (except imatinib, which is already in clinical use) whose effectiveness as chemotherapeutic agents are currently evaluated in clinical trials.

Regulation of Proteins Involved in Aerobic Respiration by p53

Collectively, these studies have laid the groundwork for understanding of how p53 regulates glycolysis and how that may lead to the identification of novel chemotherapeutic targets. Additionally, with p53-regulated proteins playing critical direct and indirect roles in regulating glycolytic energy metabolism, p53 appears to be an essential mediator of the Warburg-defined shift from aerobic respiration to a reliance on glycolysis in the malignant phenotype. Despite a large body of work outlining p53's glycolytic connections, until recently very little was known about how p53 might affect the mitochondria where proteins of the aerobic respiration pathway reside and where apoptotic cascades can be initiated. As mentioned previously, p53 not only transcriptionally regulates proteins involved in the mitochondrial apoptotic pathway such as Bax, Bcl-2, and PUMA, but also translocates to the mitochondria to facilitate apoptotic signaling (Perfettini et al. 2004; Erster and Moll 2005). Also, p53 disrupts mitochondrial membrane potential, in part, through upregulating the expression of p53AIP1 (Li, Dietz and von Harsdorf 1999; Oda et al 2000). Whether p53AIP1 affects mitochondrial proteins involved in aerobic respiration is, however, unknown. Despite an increased understanding of how p53 regulates signaling pathways of cell death from mitochondria, the elucidation of how p53 directs metabolic processes for sustaining life in the cell's powerhouse has remained more elusive.

Proteins Directly Involved in Aerobic Respiration

Cytochrome C Oxidase (COX)

Mammalian cytochrome c oxidase (COX) is responsible for catalyzing the terminal reaction of the oxidative phosphorylation chain, reducing oxygen to water and transferring protons into the mitochondrial intermembrane space (Fig. 6). COX (otherwise known as Complex IV of the respiratory chain) is currently believed to be made up of 13 core subunits, however, from the import of nuclear encoded subunits into the mitochondria to its proper assembly involves as many as 30 additional accessory proteins (Khalimonchuk and Rodel 2005). Of the multiple proteins that comprise the holoenzyme, the core COXI, COXII, and COXIII subunits are encoded by the mitochondrial genome (Capaldi 1990; Shoubridge 2001). A study performed by Okamura et al. (1999) was the first to establish a direct link between p53 and COX by showing p53 induction correlated with an upregulation of COXI subunit expression at the mRNA level. Further studies are needed, however, to elucidate the mechanism for p53-mediated COXI regulation and to evaluate its direct effect on COX function. A subsequent study by Singh and colleagues demonstrated that COXII expression was also partially dependent on p53 (Zhou, Kachhap and Singh 2003). It was shown that loss of p53 resulted in decreased

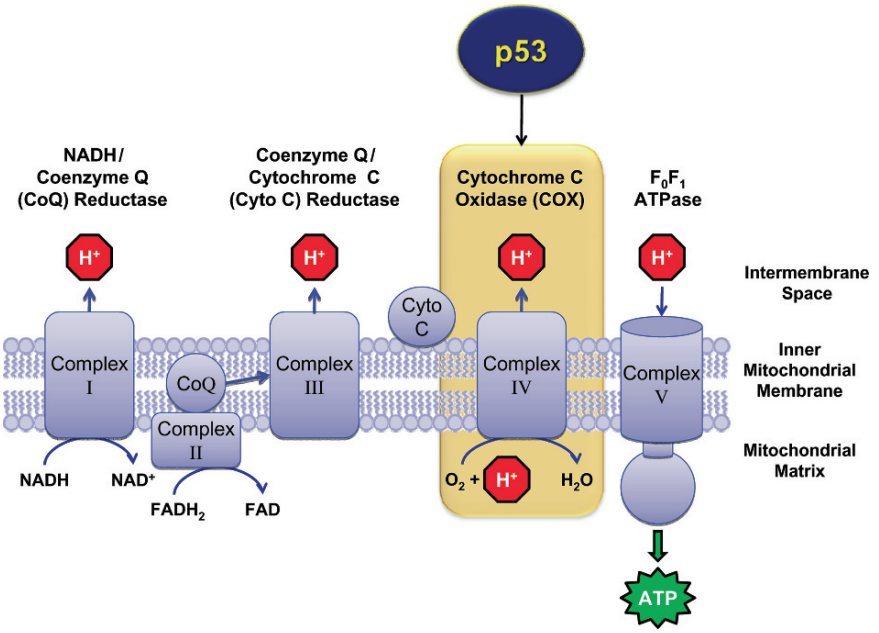

Fig. 6 The regulation of aerobic respiration by p53. The proteins of aerobic respiration (also referred to as oxidative phosphorylation or the electron transport chain) are responsible for eventually transferring electrons from NADH and $FADH_2$ produced during glycolysis and the citric acid cycle to oxygen. Generation of ATP occurs when protons (H^+) generated by Complexes I–IV are translocated to the mitochondrial matrix by Complex V (F_0F_1 ATPase). p53 has been shown to promote aerobic respiration by enhancing the expression of cytochrome c oxidase (COX) subunits (Okamura et al. 1999; Zhou et al. 2003) as well as the synthesis of cytochrome oxidase 2 (SCO2) assembly protein (Matoba et al. 2006)

COXII protein expression with concomitant decreases in COX enzyme activity in $p53^{-/-}$ cells versus isogenic p53 wild-type counterparts. Since it is well-established that p53 acts a transcription factor to regulate gene transcription and several subunits of the COX holoenzyme are encoded within nuclear DNA, it is conceivable that p53 could also regulate the expression of one of the nuclear encoded COX subunits directly, however evidence supporting such a notion is at this time lacking.

Proteins Indirectly Involved in Aerobic Respiration

Synthesis of Cytochrome C Oxidase 2 (SCO2)

Renewed interest in the relationship between p53 and aerobic respiration has been inspired by a recent study by Matoba et al. (2006). Serial analysis of gene expression (SAGE) database searches revealed that synthesis of cytochrome c oxidase

2 (SCO2) was expressed in a p53-dependent manner in a wild-type p53 expressing colon cancer cell line. Along with SCO1, SCO2 is an important mediator in the delivery of copper to the COX catalytic core, an essential step in producing the active COX holoenzyme (Horng et al. 2005). The in vivo result of direct transcriptional activation of SCO2 by p53 was enhanced oxygen consumption and ATP generation, an effect lost in $p53^{-/-}$ mice and p53-deficient colon cancer cells (Matoba et al. 2006). Additionally, p53 regulation of SCO2 was specific, as p53 expression had no effect on SCO1 levels. The novel discovery that p53 also modulates proteins such as SCO2 involved with oxidative phosphorylation provides additional means by which p53 may carry out its tumor suppressor function both as a transcription factor and through its increasingly important role in mitochondria transactions. The possibility that p53 could regulate still more proteins involved in COX function or perhaps other protein complex involved in the electron transport chain is a question that will likely be addressed in years to come. In a much broader picture, the discovery of COXI, COXII and SCO2 as p53-regulated proteins compliments the discoveries made in regards to what has been learned about p53's regulation of glycolysis.

Regulation of p53 by the Products of Glycolysis and Aerobic Respiration

The issue as to how the major products of glycolytic and aerobic respiratory pathways reciprocally affect p53 function also warrants some discussion. Interestingly, the effects the metabolites have on p53 are as complex as the system p53 regulates to catalyze them. Pyruvate, for instance, is the major metabolic product of glycolysis and has been shown to induce p53 expression (Xu and Finkel 2002). The function of ATP has been shown to extend past just simply an energy store with its ability to bind p53 at its C-terminus and promote its dissociation from DNA (Okorokov and Milner 1999). Similarly, another major product of aerobic respiration, NAD^+, appears to also be intimately linked to negative p53 regulation. Like ATP, NAD^+ also binds p53 and promotes its dissociation from p53-DNA complexes (McLure et al. 2004). Additionally, NAD^+ functions as a critical regulator of multiple p53-interacting proteins involved in (i) the negative regulation of p53 tumor suppressor function (Luo et al. 2001; Vaziri et al. 2001) (ii) protecting the cell against apoptosis via ROS (Yoon et al. 2004), and (iii) the response to DNA damage (Herceg and Wang 2001). Interestingly, although most NAD^+ is thought to reside in the mitochondria, a definitive conclusion as to whether it also translocates to the cytosol has yet to be reached. Published evidence indicates that NAD^+ does indeed transport across the mitochondrial membrane (Rustin et al. 1996; Bradshaw and Pfeiffer 2006) and therefore aerobic respiration-derived NAD^+ could affect p53 localized to the mitochondria.

Conclusions and Future Directions

There is now a renewed fervor in realizing the significance of the 80-year-old phenomenon known as the "Warburg effect." The revived interest in this area of investigation is fully justified as it is becoming increasingly important to mechanistically understand why a cancer cell loses its dependence on oxygen for energy generation and gains a preference for glycolysis. In this regard, several lines of recent evidence indicate that the tumor suppressor p53 appears to be involved in regulating this shift. The p53 tumor suppressor is one of the most extensively investigated proteins relevant to cancers. The findings that p53 is implicated in regulating the energy generating metabolic pathways should prove highly significant as drugs are being developed to target p53 and p53-controlled signaling events. It is expected that more information on how p53 regulates these essential metabolic pathways will be forthcoming that will aid in the identification of new p53-regulated targets. As more information becomes available, intriguing new possibilities for cancer treatment will also emerge.

References

Arora, K. K. and Pedersen, P. L. 1988. Functional significance of mitochondrial bound hexokinase in tumor cell metabolism. Evidence for preferential phosphorylation of glucose by intramitochondrially generated ATP. J. Biol. Chem. 263:17422–17428.

Bensaad, K., Tsuruta, A., Selak, M. A., Vidal, M. N., Nakano, K., Bartrons, R., Gottlieb, E., Vousden, K. H. 2006. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 126:107–120.

Board, M., Colquhoun, A., Newsholme, E. A. 1995. High Km glucose-phosphorylating (glucokinase) activities in a range of tumor cell lines and inhibition of rates of tumor growth by the specific enzyme inhibitor mannoheptulose. Cancer Res. 55:3278–3285.

Boren, J., Cascante, M., Marin, S., Comín-Anduix, B., Centelles, J. J., Lim, S., Bassilian, S., Ahmed, S., Lee, W. N., Boros, L. G. 2001. Gleevec (STI571) influences metabolic enzyme activities and glucose carbon flow toward nucleic acid and fatty acid synthesis in myeloid tumor cells. J. Biol. Chem. 276:37747–37753.

Bradshaw, P. C. and Pfeiffer, D. R. 2006. Loss of NAD(H) from swollen yeast mitochondria. BMC Biochem. 7:3.

Briasoulis, E., Pavlidis, N., Terret, C., Bauer, J., Fiedler, W., Schoffski, P., Raoul, J. L., Hess, D., Selvais, R., Lacombe, D., Bachmann, P., Fumoleau, P. 2003. Glufosfamide administered using a 1-hour infusion given as first-line treatment for advanced pancreatic cancer. A phase II trial of the EORTC-new drug development group. Eur. J. Cancer 39:2334–2340.

Bryson, J. M., Coy, P. E., Gottlob, K., Hay, N., Robey, R. B. 2002. Increased hexokinase activity, of either ectopic or endogenous origin, protects renal epithelial cells against acute oxidant-induced cell death. J. Biol. Chem. 277:11392–11400.

Capaldi, R. A. 1990. Structure and function of cytochrome c oxidase. Annu. Rev. Biochem. 59:569–596.

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., Green, D. R. 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303:1010–1014.

Corcoran, C. A., Huang, Y. and Sheikh, M. S. 2004. The p53 paddy wagon: COP1, Pirh2 and MDM2 are found resisting apoptosis and growth arrest. Cancer Biol. Ther. 3:721–725.

Craig, A. L., Chrystal, J. A., Fraser, J. A., Sphyris, N., Lin, Y., Harrison, B. J., Scott, M. T., Dornreiter, I., Hupp, T. R. 2007. The MDM2 ubiquitination signal in the DNA-binding domain of p53 forms a docking site for calcium calmodulin kinase superfamily members. Mol. Cell. Biol. 27:3542–3555.

Di Cosimo, S., Ferretti, G., Papaldo, P., Carlini, P., Fabi, A., Cognetti, F. 2003. Lonidamine: efficacy and safety in clinical trials for the treatment of solid tumors. Drugs Today (Barc) 39:157–174

Durany, N., Joseph, J., Campo, E., Molina, R., Carreras, J. 1997a. Phosphoglycerate mutase, 2,3-bisphosphoglycerate phosphatase and enolase activity and isoenzymes in lung, colon and liver carcinomas. Br. J. Cancer 75:969–977.

Durany, N., Joseph, J., Cruz-Sanchez, F. F., Carreras, J. 1997b. Phosphoglycerate mutase, 2,3-bisphosphoglycerate phosphatase and creatine kinase activity and isoenzymes in human brain tumours. Br. J. Cancer 76:1139–1149.

Durany, N., Joseph, J., Jimenez, O. M., Climent, F., Fernandez, P. L., Rivera, F., Carreras, J. 2000. Phosphoglycerate mutase, 2,3-bisphosphoglycerate phosphatase, creatine kinase and enolase activity and isoenzymes in breast carcinoma. Br. J. Cancer 82:20–7.

Erster, S. and Moll, U. M. 2005. Stress-induced p53 runs a transcription-independent death program. Biochem. Biophys. Res. Commun. 331:843–850.

Fang, L., Li, G., Liu, G., Lee, S. W., Aaronson, S. A. 2001. p53 induction of heparin-binding EGF-like growth factor counteracts p53 growth suppression through activation of MAPK and PI3K/Akt signaling cascades. EMBO J. 20:1931–1939.

Feng, Z., Hu, W., de Stanchina, E., Teresky, A. K., Jin, S., Lowe, S., Levine, A. J. 2007. The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. Cancer Res. 67:3043–3053.

Floridi, A., Paggi, M. G., Marcante, M. L., Silvestrini, B., Caputo, A., De Martino, C. 1981. Lonidamine, a selective inhibitor of aerobic glycolysis of murine tumor cells. J. Natl. Cancer Inst. 66:497–499.

Floridi, A., Paggi, M. G., D'Atri, S., De Martino, C., Marcante, M. L., Silvestrini, B., Caputo, A. 1981. Effect of lonidamine on the energy metabolism of Ehrlich ascites tumor cells. Cancer Res. 41:4661–4666.

Forte, M., Adelsberger-Mangan, D., and Colombini, M. 1987. Purification and characterization of the voltage-dependent anion channel from the outer mitochondrial membrane of yeast. J. Membr. Biol. 99:65–72.

Gatenby, R. A. and Gillies, R. J. 2004. Why do cancers have high aerobic glycolysis? Nat. Rev. Cancer 4:891–899.

Giaccone, G., Smit, E. F., de Jonge, M., Dansin, E., Briasoulis, E., Ardizzoni, A., Douillard, J. Y., Spaeth, D., Lacombe, D., Baron, B., Bachmann, P., Fumoleau, P.; EORTC-New Drug Development Group. 2004. Glufosfamide administered by 1-hour infusion as a second-line treatment for advanced non-small cell lung cancer; a phase II trial of the EORTC-New Drug Development Group. Eur. J. Cancer 40:667–672.

Glass-Marmor, L., Beitner, R. 1997. Detachment of glycolytic enzymes from cytoskeleton of melanoma cells induced by calmodulin antagonists. Eur. J. Pharmacol. 328:241–8.

Gottschalk, S., Anderson, N., Hainz, C., Eckhardt, S. G., Serkova, N. J. 2004. Imatinib (STI571)-mediated changes in glucose metabolism in human leukemia BCR-ABL-positive cells. Clin. Cancer Res. 10:6661–6668.

Herceg, Z. and Wang, Z. Q. 2001. Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. Mutat. Res. 477:97–110.

Horng, Y. C., Leary, S. C., Cobine, P. A., Young, F. B., George, G. N., Shoubridge, E. A., Winge, D. R. 2005. Human Sco1 and Sco2 function as copper-binding proteins. J. Biol. Chem. 280:34113–34122.

Imamura, K., Ogura, T., Kishimoto, A., Kaminishi, M., Esumi, H. 2001. Cell cycle regulation via p53 phosphorylation by a 5 -AMP activated protein kinase activator, 5-aminoimidazole- 4-carboxamide-1-beta-D-ribofuranoside, in a human hepatocellular carcinoma cell line. Biochem. Biophys. Res. Commun. 287:562–567.

Jones, R. G., Plas, D. R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M. J., Thompson, C. B. 2005. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol. Cell 18:283–293.

Kang, H. T. and Hwang, E. S. 2006. 2-Deoxyglucose: an anticancer and antiviral therapeutic, but not any more a low glucose mimetic. Life Sci. 78:1392–1399.

Karuman, P., Gozani, O., Odze, R. D., Zhou, X. C., Zhu, H., Shaw, R., Brien, T. P., Bozzuto, C. D., Ooi, D., Cantley, L. C., Yuan, J. 2001. The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death. Mol. Cell 7:1307–1319.

Katajisto, P., Vallenius, T., Vaahtomeri, K., Ekman, N., Udd, L., Tiainen, M., Makela, T. P. 2007. The LKB1 tumor suppressor kinase in human disease. Biochim. Biophys. Acta. 1775:63–75.

Khalimonchuk, O. and Rodel, G. 2005. Biogenesis of cytochrome c oxidase. Mitochondrion 5:363–388.

Kondoh, H., Lleonart, M. E., Gil, J., Wang, J., Degan, P., Peters, G., Martinez, D., Carnero, A., Beach, D. 2005. Glycolytic enzymes can modulate cellular life span. Cancer Res. 65:177–185.

Lavin, M. F. and Gueven, N. 2006. The complexity of p53 stabilization and activation. Cell Death Differ. 13:941–950.

Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E., George, D. L. 2004. Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat. Cell Biol. 6:443–450.

Li, P. F., Dietz, R. and von Harsdorf, R. 1999. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. EMBO J. 18:6027–6036.

Lim, Y. P., Lim, T. T., Chan, Y. L., Song, A. C., Yeo, B. H., Vojtesek, B., Coomber, D., Rajagopal, G., Lane, D. 2007. The p53 knowledgebase: an integrated information resource for p53 research. Oncogene 26:1517–1521.

Ludes-Meyers, J. H., Subler, M. A., Shivakumar, C. V., Munoz, R. M., Jiang, P., Bigger, J. E., Brown, D. R., Deb, S. P., Deb, S. 1996. Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol. Cell. Biol. 16:6009–6019.

Luo, J., Nikolaev, A. Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., Gu, W. 2001. Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell 107:137–148.

Luo, Z., Saha, A. K., Xiang, X., Ruderman, N. B. 2005. AMPK, the metabolic syndrome and cancer. Trends Pharmacol. Sci. 26:69–76.

Macheda, M. L., Rogers, S. and Best, J. D. 2005. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J. Cell Physiol. 202:654–662.

Majewski, N., Nogueira, V., Robey, R. B., Hay, N. 2004a. Akt inhibits apoptosis downstream of BID cleavage via a glucose-dependent mechanism involving mitochondrial hexokinases. Mol. Cell. Biol. 24:730–740.

Majewski, N., Nogueira, V., Bhaskar, P., Coy, P. E., Skeen, J. E., Gottlob, K., Chandel, N. S., Thompson, C. B., Robey, R. B., Hay, N. 2004b. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. Mol. Cell 16:819–830.

Mathupala, S. P., Rempel, A. and Pedersen, P. L. 1997a. Aberrant glycolytic metabolism of cancer cells: a remarkable coordination of genetic, transcriptional, post-translational, and mutational events that lead to a critical role for type II hexokinase. J. Bioenerg. Biomembr. 29:339–343.

Mathupala, S. P., Heese, C. and Pedersen, P. L. 1997b. Glucose catabolism in cancer cells. The type II hexokinase promoter contains functionally active response elements for the tumor suppressor p53. J. Biol. Chem. 272:22776–22780.

Mathupala, S. P., Ko, Y. H. and Pedersen, P. L. 2006. Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. Oncogene 25:4777–4786.

Matoba, S., Kang, J. G., Patino, W. D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P. J., Bunz, F., Hwang, P. M. 2006. p53 regulates mitochondrial respiration. Science 312:1650–1653.

McGarrity, T. J. and Amos, C. 2006. Peutz-Jeghers syndrome: clinicopathology and molecular alterations. Cell. Mol. Life Sci. 63:2135–2144.

McLure, K. G., Takagi, M. and Kastan, M. B. 2004. NAD+ modulates p53 DNA binding specificity and function. Mol. Cell. Biol. 24:9958–9967.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., Moll, U. M. 2003. p53 has a direct apoptogenic role at the mitochondria. Mol. Cell 11:577–590.

Mohanti, B. K., Rath, G. K., Anantha, N., Kannan, V., Das, B. S., Chandramouli, B. A., Banerjee, A. K., Das, S., Jena, A., Ravichandran, R., Sahi, U. P., Kumar, R., Kapoor, N., Kalia, V. K., Dwarakanath, B. S., Jain, V. 1996. Improving cancer radiotherapy with 2-deoxy-D-glucose: phase I/II clinical trials on human cerebral gliomas. Int. J. Radiat. Oncol. Biol. Phys. 35:103–111.

Nakashima, R. A., Mangan, P. S., Colombini, M., Pedersen, P. L. 1986. Hexokinase receptor complex in hepatoma mitochondria: evidence from *N*,*N*'-dicyclohexylcarbodiimide-labeling studies for the involvement of the pore-forming protein VDAC. Biochemistry 25:1015–1021.

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., Taya, Y. 2000. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 102:849–862.

Okamura, S., Ng, C. C., Koyama, K., Takei, Y., Arakawa, H., Monden, M., Nakamura, Y. 1999. Identification of seven genes regulated by wild-type p53 in a colon cancer cell line carrying a well-controlled wild-type p53 expression system. Oncol. Res. 11:281–285.

Okar, D. A., Manzano, A., Navarro-Sabate, A., Riera, L., Bartrons, R., Lange, A. J. 2001. PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem. Sci. 26:30–35.

Oren, M. 2003. Decision making by p53: life, death and cancer. Cell Death Differ. 10:431–442.

Okorokov, A. L. and Milner, J. 1999. An ATP/ADP-dependent molecular switch regulates the stability of p53-DNA complexes. Mol. Cell. Biol. 19:7501–7510.

Ortega-Camarillo, C., Guzman-Grenfell, A. M., Garcia-Macedo, R., Rosales-Torres, A. M., Avalos-Rodriguez, A., Duran-Reyes, G., Medina-Navarro, R., Cruz, M., Diaz-Flores, M., Kumate, J. 2006. Hyperglycemia induces apoptosis and p53 mobilization to mitochondria in RINm5F cells. Mol. Cell. Biochem. 281:163–171.

Parry, D. M. and Pedersen, P. L. 1983. Intracellular localization and properties of particulate hexokinase in the Novikoff ascites tumor. Evidence for an outer mitochondrial membrane location. J. Biol. Chem. 258:10904–10912.

Pastorino, J. G., Shulga, N. and Hoek, J. B. 2002. Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. J. Biol. Chem. 277:7610–7618.

Pelicano, H., Martin, D. S., Xu, R. H., Huang, P. 2006. Glycolysis inhibition for anticancer treatment. Oncogene 25:4633–4646.

Penso, J. and Beitner, R. 1998. Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells. Eur. J. Pharmacol. 342:113–117.

Perfettini, J. L., Kroemer, R. T. and Kroemer, G. 2004. Fatal liaisons of p53 with Bax and Bak. Nat. Cell Biol. 6:386–388.

Petitjean, A., Achatz, M. I., Borresen-Dale, A. L., Hainaut, P., Olivier, M. 2007. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene 26:2157–2165.

Prives, C. and Hall, P. A. 1999. The p53 pathway. J. Pathol. 187:112–126.

Royds, J. A. and Iacopetta, B. 2006. p53 and disease: when the guardian angel fails. Cell Death. Differ. 13:1017–1026.

Ruiz-Lozano, P., Hixon, M. L., Wagner, M. W., Flores, A. I., Ikawa, S., Baldwin, A. S. Jr., Chien, K. R., Gualberto, A. 1999. p53 is a transcriptional activator of the muscle-specific phosphoglycerate mutase gene and contributes in vivo to the control of its cardiac expression. Cell Growth Differ. 10:295–306.

Rustin, P., Parfait, B., Chretien, D., Bourgeron, T., Djouadi, F., Bastin, J., Rotig, A., Munnich, A. 1996. Fluxes of nicotinamide adenine dinucleotides through mitochondrial membranes in human cultured cells. J. Biol. Chem. 271:14785–14790.

Sanchez-Cespedes, M. 2007. A role for LKB1 gene in human cancer beyond the Peutz-Jeghers syndrome. Oncogene 26(57):7825–7832.

Schwartzenberg-Bar-Yoseph, F., Armoni, M. and Karnieli, E. 2004. The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. Cancer Res. 64:2627–2633.

Seker, H., Bertram, B., Bürkle, A., Kaina, B., Pohl, J., Koepsell, H., Wiesser, M. 2000. Mechanistic aspects of the cytotoxic activity of glufosfamide, a new tumour therapeutic agent. Br. J. Cancer 82:629–634.

Selivanova, G. and Wiman, K. G. 2007. Reactivation of mutant p53: molecular mechanisms and therapeutic potential. Oncogene 26:2243–2254.

Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., Cantley, L. C. 2004. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc. Natl. Acad. Sci. USA 101:3329–3335.

Shaw, R. J., Lamia, K. A., Vasquez, D., Koo, S. H., Bardeesy, N., Depinho, R. A., Montminy, M., Cantley, L. C. 2005. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science 310:1642–1646.

Shoubridge, E. A. 2001. Cytochrome c oxidase deficiency. Am. J. Med. Genet. 106:46–52.

Singh, D., Banerji, A. K., Dwarakanath, B. S., Tripathi, R. P., Gupta, J. P., Mathew, T. L., Ravindranath, T., Jain, V. 2005. Optimizing cancer radiotherapy with 2-deoxy-d-glucose dose escalation studies in patients with glioblastoma multiforme. Strahlenther Onkol. 181:507–514.

Smith, T. A. 2000. Mammalian hexokinases and their abnormal expression in cancer. Br. J. Biomed. Sci. 57:170–178.

Spicer, J. and Ashworth, A. 2004. LKB1 kinase: master and commander of metabolism and polarity. Curr. Biol. 14:R383–R385.

Tian, M., Zhang, H., Higuchi, T., Oriuchi, N., Nakasone, Y., Takata, K., Nakajima, N., Mogi, K., Endo, K. 2005. Hexokinase-II expression in untreated oral squamous cell carcinoma: comparison with FDG PET imaging. Ann. Nucl. Med. 194:335–338.

Vassilev, L. T. 2007. MDM2 inhibitors for cancer therapy. Trends Mol. Med. 13:23–31.

Vaziri, H., Dessain, S. K., Ng Eaton, E., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L., Weinberg, R. A. 2001. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 107:149–159.

Veyhl, M., Wagner, K., Volk, C., Gorboulev, V., Baumgarten, K., Weber, W. M., Schaper, M., Bertram, B., Wiessler, M., Koepsell, H. 1998. Transport of the new chemotherapeutic agent beta-D-glucosylisophosphoramide mustard (D-19575) into tumor cells is mediated by the Na+-D-glucose cotransporter SAAT1. Proc. Natl. Acad. Sci. USA 95:2914–2919.

Vogelstein, B., Lane, D. and Levine, A. J. 2000. Surfing the p53 network. Nature 408:307–310.

Warburg, O. 1956. On respiratory impairment in cancer cells. Science 124:269–270.

Wei, C., Amos, C. I., Stephens, L. C., Campos, I., Deng, J. M., Behringer, R. R., Rashid, A., Frazier, M. L. 2005. Mutation of Lkb1 and p53 genes exert a cooperative effect on tumorigenesis. Cancer Res. 65:11297–11303.

Wick, A. N., Drury, D. R. and Morita, T. N. 1955. 2-Deoxyglucose; a metabolic block for glucose. Proc. Soc. Exp. Biol. Med. 89:579–582.

Wilson, J. E. and Chung, V. 1989. Rat brain hexokinase: further studies on the specificity of the hexose and hexose 6-phosphate binding sites. Arch. Biochem. Biophys. 269:517–525.

Wu, C., Okar, D. A., Stoeckman, A. K., Peng, L. J., Herrera, A. H., Herrera, J. E., Towle, H. C., Lange, A. J. 2004. A potential role for fructose-2,6-bisphosphate in the stimulation of hepatic glucokinase gene expression. Endocrinology 145:650–658.

Xu, D. and Finkel, T. 2002. A role for mitochondria as potential regulators of cellular life span. Biochem. Biophys. Res. Commun. 294:245–248.

Xu, R. H., Pelicano, H., Zhou, Y., Carew, J. S., Feng, L., Bhalla, K. N., Keating, M. J., Huang, P. 2005. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res. 65:613–621.

Yoon, K. A., Nakamura, Y. and Arakawa, H. 2004. Identification of ALDH4 as a p53-inducible gene and its protective role in cellular stresses. J. Hum. Genet. 49:134–140.

Zeng, P. Y. and Berger, S. L. 2006. LKB1 is recruited to the p21/WAF1 promoter by p53 to mediate transcriptional activation. Cancer Res. 66:10701–10708.

Zhou, S., Kachhap, S. and Singh, K. K. 2003. Mitochondrial impairment in p53-deficient human cancer cells. Mutagenesis 18:287–292.

Mitochondrial Tumor Suppressors

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Introduction

Cancer cells have an energy metabolism that is distinct from normal cells in that they preferentially use aerobic glycolysis (Gatenby and Gillies 2004). This feature of cancer cells were first described by Warburg, who demonstrated that although normal cells use glycolysis under hypoxia (low oxygen tension) to generate energy, cancer cells use glycolysis even when oxygen is available (Warburg 1956). Aerobic glycolysis is thought to be one of the hallmarks of aggressive cancer (Gatenby and Gillies 2004). Whether transition to aerobic glycolysis is a cause or consequence of the neoplastic transformation is unclear. In other terms, it is unknown whether the transition to aerobic glycolysis occurs *after* the crucial molecular steps of tumorigenesis take place (i.e., a tumor promoter role) or it is a mandatory proximal step that has to occur *before* tumorigenesis (i.e., a tumor initiator role). Recent studies suggest that molecular bases of the Warburg effect are caused by multiple mechanisms that commonly lead to acquisition of an ability to upregulate glycolytic pathways in cancer cells (Gatenby and Gillies 2004).

The cellular energy production by ATP generation in the presence of oxygen depends on an intact Krebs cycle and the electron transport chain, which sequentially transfers electrons extracted from the reducing elements through the mitochondrial complexes, generating an electrochemical gradient through proton pumping. Molecular oxygen acts as the terminal acceptor of the electrons. The electrochemical gradient across the inner mitochondrial membrane is converted into high energy phosphate bonds in the ATP molecules. This complex process of ATP generation by the mitochondrial complexes is also known as oxidative phosphorylation (OXPHOS) (Wallace 2005). Recent discoveries indicate that inactivation of certain Krebs cycle

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enzymes by DNA mutations in two familial cancer syndromes provides unequivocal evidence that primary abnormalities in mitochondria can cause cancer.

Mitochondria

Mitochondria are essential organelles for energy production. Each cell may contain thousands of copies of mitochondrial DNA (mtDNA), a circular 16.5 kb genome, within their mitochondria. Of the 1,500 proteins present in the mitochondria, approximately 97.5% are encoded by the nuclear genome, translated in the cytoplasm, and transported to mitochondria. The proteins encoded by the mtDNA include a subset of OXPHOS proteins, ribosomal RNAs, and transfer RNAs. The mtDNA encodes 7 of the 46 polypeptides of complex I, one of the 11 polypeptides of complex III, 3 of the 13 polypeptides of complex IV, and 2 of the 16 proteins of complex V. All four subunits of complex II are encoded by the nuclear genome (Wallace 2005).

The electrons generated by food oxidation are transferred to Complexes I (NADH:ubiquinone oxidoreductase) and II (succinate:ubiquinone oxidoreductase; succinate dehydrogenase (SDH)), which subsequently transfer them to ubiquinone (coenzyme Q). The electrons are then transferred sequentially to complex III (ubiquinole;cytochrome c oxidoreductase), cytochrome c, and finally to complex IV (cytochrome oxidase), which transfer them to molecular oxygen, the terminal electron acceptor. The electron transfer chain (ETC) process is coupled to proton (H^+) pumping across the inner mitochondrial membrane and is performed by the complexes I, III, and IV. The electrochemical gradient generated by the proton pumping is used by complex V to synthesize ATP (Saraste 1999). In addition to its role in energy production, mitochondria generate significant quantities of reactive oxygen species (ROS) and regulate apoptosis.

Role of Mitochondria in Cancer

Many studies have implicated mitochondria in the pathogenesis of cancer through identification of somatic mtDNA mutations. mtDNA mutations have been identified in a variety of tumors, including, but not limited to, breast, brain, colorectal, gastric, pancreatic, prostate, thyroid, ovarian, acute lymphoblastic leukemia, and myelodysplastic syndrome (Brandon, Baldi and Wallace 2006). Notably, most of these mtDNA mutations are somatic and thought to occur during the process of tumorigenesis. Recently, it has been suggested that some of the somatic mtDNA mutations reported in common tumors could represent sample contamination or mix-up because these mutations correspond exactly to certain populational haplotypes (Salas et al. 2005). Nevertheless, *bona fide* somatic mutations occur in constitutional mtDNA during tumorigenesis (Zanssen and Schon 2005). Although recent functional studies have

provided further evidence that certain somatic mtDNA mutations could indeed promote tumorigenesis (Gallardo et al. 2006; Brandon et al. 2006), genetic evidence showing cosegregation of inherited mtDNA mutations with cancer has yet to be obtained in nuclear families. Role of mtDNA somatic mutations in the process of tumorigenesis would be further strengthened if somatic mutations are identified in the *nuclear-encoded* mitochondrial subunits of the OXPHOS complexes. Thus, extant data raise the possibility that certain mtDNA mutations observed in common tumors might not be causally related to tumor pathogenesis, and they might become prominent because of genetic drift thought to occur during the chaotic proliferation of cancer cells (Coller et al. 2001).

The recent discovery of mutations in the nuclear genes encoding succinate dehydrogenase (SDH) and fumarate hydratase (FH) or fumarase in hereditary paraganglioma (PGL) and hereditary leiomyomatosis/renal cell cancer (HLRCC) syndromes, respectively (Baysal et al. 2000; Tomlinson et al. 2002) has provided a definitive proof that primary inherited abnormalities in mitochondria can cause tumors.

Krebs Cycle Enzymes and Inherited Tumor Susceptibility

SDH and FH catalyze two subsequent reactions in Krebs cycle (Tricarboxylic acid cycle, citric acid cycle). SDH oxidizes succinate to fumarate and is thought to be present only in the mitochondria. SDH is composed of four distinct subunits (i.e., a heterotetramere) encoded by the nuclear genome. The three dimensional structures of SDH have been recently characterized in *Escherichia coli* and porcine heart (Yankovskaya et al. 2003; Sun et al. 2005). The hydrophilic catalytic subunits, flavoprotein (Fp) and iron–sulfur (Ip) subunits, are encoded by the *SDHA* and *SDHB* genes, respectively. Fp and Ip harbors Flavin Adenine Dinucleotide (FAD) and three distinct iron-sulfur clusters, respectively. The hydrophobic large (cybL) and small (cybS) subunits span the inner mitochondrial membrane and they are encoded by the *SDHC* and *SDHD* genes, respectively. The cybL and cybS subunits sandwich a heme *b* moiety. The electrons generated during the oxidation of succinate to fumarate are first transferred to the covalently bound FAD moiety in Fp subunit, then to the iron–sulfur clusters in the Ip subunit, and finally to the ubiquinone, which is then fed into the electron transport chain (Cecchini 2003). The heme is present in all eukaryotic SDH enzymes, yet its precise function is unclear. Recent studies suggest that the heme is not absolutely required for catalysis and enzyme assembly, but may play a role in reducing superoxide production and/or facilitating catalysis (Oyedotun et al. 2007; Tran et al. 2007).

In the next reaction of the Krebs cycle, fumarate is hydrated to malate by FH in a reversible reaction. FH is present in the mitochondrial matrix as well as in cytoplasm of Metazoa, Plantae, and Fungi. A single FH gene harbors two unique start sites encoding for the mitochondrial and the cytosolic forms of fumarase (Suzuki et al. 1989). Fumarase is a tetramer of four identical polypeptide chains consisting

of 467 amino acids. Unlike SDH, FH contains no prosthetic groups (Gottlieb and Tomlinson 2005).

Before the positional cloning studies identified heterozygous germ line mutations in the SDH and FH genes in familial cancer syndromes, inborn deficiencies of these enzymes were linked to metabolic syndromes. The homozygous or compound heterozygous mutations that markedly reduce SDH or FH enzyme activities are associated with severe neurological impairment in early childhood (Bourgeron et al. 1994, 1995). SDH inborn deficiency leads to encephalopathy, myopathy, neuropathy, and Leigh Syndrome, whereas FH inborn deficiency can cause progressive encephalopathy, dystonia, leucopenia, and neutropenia. In the light of these findings, it is surprising that heterozygous mutations in these genes could lead to tumor development. The heterozygous germ line mutations in the SDH and FH tumor susceptibility genes are followed by somatic mutations targeting the remaining normal copy indicating that these mitochondrial enzymes are classical tumor suppressor genes in the tissue types transformed into tumors. Thus haploinsufficiency of these enzymes appears to be well tolerated in other tissues.

Mechanisms of Pathogenesis in PGL

PGL is characterized by the development of highly vascular tumors in the paraganglionic system (Baysal 2002). The paraganglionic system is composed of many clusters of small tissues and organs which share a common embryological origin and histological structure and is distributed from the skull base to the pelvic floor. The major paraganglionic organs are the carotid body (CB) and the adrenal medulla in the adult and the organ of Zuckerkandl (paraaortic abdominal paraganglia) in the embryo. Mutations in the *SDHD* gene were first discovered by genetic mapping techniques on chromosome band 11q23 using extended PGL families (Baysal et al. 2000). The discovery of *SDHD* mutations led to identification of mutations in the other subunit genes *SDHB* on chromosome band 1p35 (PGL type 4, PGL4) (Astuti et al. 2001) and *SDHC* on chromosome band 1q23 (PGL3) (Niemann and Muller 2000) in PGL families.

More than 100 mutations have been reported in PGL families since the first mutations were identified in *SDHD* gene (Bayley et al. 2005). The majority of PGL mutations occur in the *SDHD* or *SDHB* genes, whereas *SDHC* mutations are uncommon (Baysal et al. 2002; Neumann et al. 2002). Thus far, no *SDHA* mutations have been detected in PGL. The tumor development among *SDHD* mutation carriers occurs only if the mutation is transmitted paternally (Van der Mey et al. 1989; Baysal et al. 2000). Maternal transmission of an *SDHD* mutation does not cause tumor development, suggesting that genomic imprinting silences the *SDHD* gene upon maternal transmission. No parent-of-origin effects were observed in the transmission of *SDHB* and *SDHC* mutations, suggesting that a locus specific effect at chromosome band 11q23 operates on the *SDHD* gene.

Mutations in *SDHD* or *SDHB* genes may cause tumor development in any anatomical component of the paraganglionic system. However, mutations in *SDHD* are more common among subjects with head and neck paragangliomas (HNPs) (Astrom et al. 2003; Neumann et al. 2004). HNPs are usually benign and slow-growing tumors that may impinge upon critical anatomical structures in the head and neck. A germ line mutation in *SDHD*, *SDHB*, or *SDHC* genes is expected to be present in more than 15% of unselected subjects with HNPs (Baysal et al. 2002; Neumann et al. 2002; Schiavi et al. 2005). The carotid body, a small oxygen-sensing organ located at the bifurcation of the common carotid artery in the neck, is the most common HNP tumor location (Astrom et al. 2003). Mutations in *SDHB* are more common among subjects with abdominal paragangliomas and pheochromocytomas (i.e., hormonally active paragangliomas) (Neumann et al. 2002; Amar et al. 2005). Nearly 8% of nonsyndromic pheochromocytomas carry germ line mutations in *SDHB* or less commonly in the *SDHD* genes. The abdominal paragangliomas caused by *SDHB* mutations are often malignant (Neumann et al. 2004; Timmers et al. 2007). Recent data suggest that 30% of all malignant pheochromocytomas may be caused by a germ line *SDHB* mutation (Brouwers et al. 2006). In contrast, malignant paragangliomas associated with *SDHD* mutations are uncommon (Astrom et al. 2003). Because *SDHD* mutations are associated with HNPs and *SDHB* mutations with abdominal paragangliomas, an important question is whether malignancy is associated with the mutated gene or with the anatomical location.

Because sporadic CB paragangliomas that develop as a result of chronic hypoxic stimulation at high altitudes (Arias-Stella and Valcarcel 1976) and familial paragangliomas caused by *SDHD* mutations are markedly similar, a role for SDH in oxygen sensing was suggested (Baysal et al. 2000). An analysis of gene–environment interaction among *SDHD* mutation carriers suggested that higher altitudes were associated with increased clinical severity, decreased proportion of sporadic cases with *SDHD* germ line mutations, and decreased likelihood of *SDHD* founder mutations in the population, supporting the model that PGL mutations lead to chronic hypoxic stimulation in the paraganglionic tissues (Astrom et al. 2003). The homozygous loss of the *SDHD* gene in mouse is incompatible with development and mice that are heterozygous for the *SDHD* mutation do not develop CB tumors but show an increased baseline hypoxic discharge in the CB in normoxia (Piruat et al. 2004), consistent with the model that *SDHD* mutations lead to constitutive hypoxic stimulation in the paraganglia (Baysal et al. 2000). The mouse model suggested that reduction of SDH dosage leads to increased production of superoxide radicals. Importantly, increased superoxide production upon SDH mutations has been suggested in several other models including the *Caenorhabditis elegans* (*C. elegans*) mev-1 model caused by an *SDHC* homozygous mutation (Ishii et al. 1998) and yeast models of *SDHB* knockdown and ubiquinone binding site mutants (Szeto et al. 2007; Smith et al. 2007). Collectively, results from these models suggest that loss of SDH function causes increased reactive oxygen species (ROS) production, which leads to persistent hypoxic stimulation of the paraganglionic tissues.

Another potential link between PGL and stimulation of hypoxic response pathways has been suggested through accumulation of succinate as result of SDH inactivation. It has been demonstrated that succinate inhibits hypoxia inducible factor (HIF) prolyl hydroxylases (HPHs) (Selak et al. 2005). HIF hydroxylases are a recently discovered group of enzymes that use molecular oxygen to hydroxylate HIF1α and facilitate its degradation in normoxia (Schofield and Ratcliffe 2004; Kaelin 2005). The degradation of hydroxylated HIF1α is mediated through binding of pVHL, which is encoded by the gene mutated in Von Hippel Lindau disease. pVHL is part of an E3 ubiquitin ligase complex that targets the hydroxylated HIF1α subunits for degradation. Notably, intermediary metabolites of the Krebs cycle, α-ketoglutarate (2-oxoglutarate) and succinate are also substrates and products, respectively, of HPHs. The inhibition of HPHs by the increased succinate levels and the subsequent stabilization of HIF1α could partially be responsible for the activation of hypoxic factors and the highly vascular nature of paraganglioma tumors. Further studies on the impact of succinate accumulation raise the possibility that pathways other than HIF1α could play a role in tumor pathogenesis. These pathways include inhibition of apoptosis (Lee et al. 2005) and histone demethylation (Smith, Janknecht and Maher 2007), mechanisms that may impact regulation of gene expression relevant for PGL tumorigenesis. In summary, recent findings suggest that ROS and succinate may jointly play a role in tumor pathogenesis by activating the hypoxic response pathways in PGL.

Mechanism of Pathogenesis in HLRCC

Hereditary leiomyomatosis and renal cell cancer (HLRCC) is an autosomal dominant disorder characterized by the development of smooth-muscle tumors of the skin and uterus and renal cancer (Kiuru and Launonen 2004). Notably, although FH and SDH catalyze the two successive steps in the Krebs cycle, there is no overlap among the component tumors in HLRCC and PGL. Although cutaneous leiomyomas (piloleiomyomas), benign tumors arising from the arrector pili muscle of hair follicles, are rare, the uterine leiomyomas (fibroids) are the most common benign tumors in women with a lifetime incidence of 70%. Analyses of mutations in the FH gene located on chromosome band 1q43 suggest that occult germ line mutations contribute to pathogenesis of only a small fraction of the common sporadic form of uterine leiomyomas (Kiuru et al. 2002; Barker et al. 2002). Despite the small contribution of FH mutations to sporadic leiomyomas, understanding the mechanism of tumorigenesis in HLRCC may also provide insights for pathogenesis of the common leiomyomas.

The penetrance for skin and uterine leiomyomas in HLRCC could be as high as 75% and 100%, respectively. The female FH mutation carriers develop uterine leiomyomas before they reach age 45 and skin leiomyomas may develop in most carriers before they reach age 40 (Alam et al. 2005; Wei et al. 2006). The penetrance of kidney tumors is estimated from 10% to 62% of germ line FH

mutation carriers, suggesting that renal cancer predisposition is determined by additional genetic or environmental factors among FH mutation carriers. Genotype–phenotype correlations to determine whether certain mutations predispose to certain type of component tumors have yet to be identified in HLRCC.

The mechanism of tumorigenesis in HLRCC is even more enigmatic than PGL, because no obvious physiopathologic link exists between FH mutations and transformation in smooth muscle and kidney tumors. It has been biochemically demonstrated that fumarate accumulation by FH downregulation leads to inhibition of hypoxia inducible factor (HIF) prolyl hydroxylases (HPHs). Although overexpression of hypoxia-inducible genes was observed in HLRCC leiomyomas (Pollard et al. 2005), but not in the sporadic leiomyomas, genome wide gene expression profiling in HLRCC leiomyomas indicates induction of glycolytic enzymes rather than hypoxia-inducible genes (Vanharanta et al. 2006). In contrast, gene expression profiles of paraganglioma tumors caused by SDH mutations strongly indicate hypoxic pathway activation (Dahia et al. 2005). Thus in HLRCC, there is a discrepancy between molecular studies, which suggest induction of hypoxia inducible pathways by *in vitro* biochemical assays and in a mouse model where inactivation of the FH gene in kidney causes renal cysts (Pollard et al. 2007), and the gene expression profiles of HLRCC tumors and the epidemiological evidence, which do not suggest a role for hypoxia in the causation of leiomyomas. Therefore it is unclear whether fumarate inhibition of HPHs, and subsequent activation of hypoxia-inducible pathways is the driving mechanism of pathogenesis in HLRCC tumors. It is conceivable that the etiology of the sporadic leiomyomas could suggest a potential mechanism in HLRCC, similar to the original proposal that suggested defective oxygen-sensing in PGL on the basis of the association between sporadic paragangliomas and chronic hypoxic stimulation.

Certain female reproductive factors such as early menarche, nulliparity, late reproductive years, obesity, African-American ancestry, Tamoxifen use have been associated with the risk of uterine leiomyomas, suggesting that increased overall estrogen exposure or more menstruations could increase risk of these common tumors (Parker 2007). It is plausible that the genetic defect in HLRCC could constitutively activate physiological pathways in smooth muscle cells relevant for these epidemiological risk factors facilitating leiomyoma development. For example, if contraction of smooth muscle cells is a final common pathway for certain leiomyoma risk factors, the smooth muscle contraction could be overactivated by heterozygous FH mutations, leading to tumorigenesis.

Notably, fumarate, which accumulates in HLRCC, is also a by-product of the urea cycle (Shambaugh 1977). The urea cycle converts ammonia produced by degradation of the amino acids into urea. The cytosolic isoform of the FH converts the fumarate produced by the urea cycle to malate. The shedding and degradation of endometrium cyclically occurring at the end of each menstrual cycle could lead to an increased rate of amino acid degradation and subsequently increased fumarate production by the urea cycle. If fumarate generated by the urea cycle triggers uterine smooth muscle cell contraction, then chronically high fumarate levels in HLRCC could lead to constitutive contractile signaling that could finally lead to

leiomyoma development. If fumarate produced by the urea cycle has a critical role in leiomyoma development in HLRCC, inhibition of the urea cycle by activating latent biological pathways for ammonia disposal could reduce the risk of leiomyoma development in HLRCC.

Summary and Conclusions

The surprising discoveries that link inherited mutations in the Krebs cycle enzymes SDH and FH to tumor development have expanded our understanding of tumorigenesis by providing the first causative associations between mitochondrial dysfunction and neoplasia. The underlying mechanisms of mitochondrial tumorigenesis are beginning to be understood, although many fundamental questions remain unanswered (e.g., why do only certain tissues develop neoplasia although these genes expressed in all tissues as part of oxidative phosphorylation pathways?). The mutations in SDH could disrupt oxygen sensing and lead to constitutive hypoxic signaling in paraganglia whereas mutations in FH might lead to constitutive activation of an unknown distinct physiological signaling pathway, such as contraction, in smooth muscle cells through fumarate accumulation (Fig. 1). These physiological pathways are likely to be overactivated by heterozygous germ line mutations in SDH and FH genes. The complete somatic inactivation of the SDH and FH by somatic loss of the normal nonmutated alleles, as occurs in most classical tumor suppressor genes, is predicted to interrupt the Krebs cycle in the tumors of both diseases. Thus it can be concluded that the component tumors in PGL and HLRCC thrive in the absence of

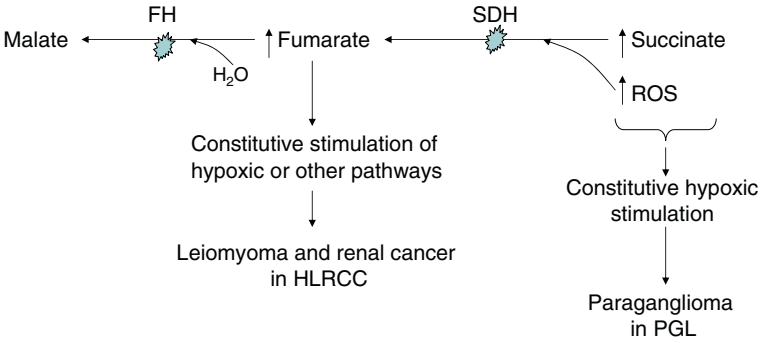

Fig. 1 Models for pathogenesis of PGL and HLRCC syndromes. In the Krebs cycle, succinate is oxidized to fumarate by succinate dehydrogenase (SDH), then fumarate is hydrated to malate by fumarate hydratase (FH). Fumarate is also produced by the urea cycle and is converted to malate by the cytoplasmic FH. Mutations (shown by explosion signs) in SDH in PGL lead to accumulation of succinate and reactive oxygen species (ROS), which induce chronic hypoxic signaling and paraganglioma tumor development in PGL. Mutations in FH lead to accumulation of fumarate, which then triggers hypoxic or other unknown biochemical pathways important in smooth muscle and kidney metabolism that causes neoplastic transformation of HLRCC

functional Krebs cycle and oxidative phosphorylation. Thus PGL and HLRCC provide proof that inborn mitochondrial defects can cause tumors and explain the molecular basis of Warburg's observations in certain tumors that are rendered genetically defective in the essential mitochondrial enzymes SDH and FH.

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References

Alam, N. A., Barclay, E., Rowan, A. J., Tyrer, J. P., Calonje, E., Manek, S., Kelsell, D., Leigh, I., Olpin, S., and Tomlinson, I. P. 2005. Clinical features of multiple cutaneous and uterine leiomyomatosis: An underdiagnosed tumor syndrome. Arch. Dermatol. 141: 199–206.

Amar, L., Bertherat, J., Baudin, E., Ajzenberg, C., Bressac-de Paillerets, B., Chabre, O., Chamontin, B., Delemer, B., Giraud, S., Murat, A., Niccoli-Sire, P., Richard, S., Rohmer, V., Sadoul, J. L., Strompf, L., Schlumberger, M., Bertagna, X., Plouin, P. F., Jeunemaitre, X., and Gimenez-Roqueplo, A. P. 2005. Genetic testing in pheochromocytoma or functional paraganglioma. J. Clin. Oncol. 23: 8812–8818.

Arias-Stella, J. and Valcarcel, J. 1976. Chief cell hyperplasia in the human carotid body at high altitudes; physiologic and pathologic significance. Hum. Pathol. 7: 361–373.

Astrom, K., Cohen, J. E., Willett-Brozick, J. E., Aston, C. E., and Baysal, B. E. 2003. Altitude is a phenotypic modifier in hereditary paraganglioma type 1: Evidence for an oxygen-sensing defect. Hum. Genet. 113: 228–237.

Astuti, D., Latif, F., Dallol, A., Dahia, P. L., Douglas, F., George, E., Skoldberg, F., Husebye, E. S., Eng, C., and Maher, E. R. 2001. Gene mutations in the succinate dehydrogenase subunit *SDHB* cause susceptibility to familial pheochromocytoma and to familial paraganglioma. Am. J. Hum. Genet. 69: 49–54.

Barker, K. T., Bevan, S., Wang, R., Lu, Y. J., Flanagan, A. M., Bridge, J. A., Fisher, C., Finlayson, C. J., Shipley, J., and Houlston, R. S. 2002. Low frequency of somatic mutations in the FH/multiple cutaneous leiomyomatosis gene in sporadic leiomyosarcomas and uterine leiomyomas. Br. J. Cancer 87: 446–448.

Bayley, J. P., Devilee, P., and Taschner, P. E. 2005. The SDH mutation database: An online resource for succinate dehydrogenase sequence variants involved in pheochromocytoma, paraganglioma and mitochondrial complex II deficiency. BMC Med. Genet. 6: 39.

Baysal, B. E. 2002. Hereditary paraganglioma targets diverse paraganglia. J. Med. Genet. 39: 617–622.

Baysal, B. E., Ferrell, R. E., Willett-Brozick, J. E., Lawrence, E. C., Myssiorek, D., Bosch, A., van der, M. A., Taschner, P. E., Rubinstein, W. S., Myers, E. N., Richard, C. W., III, Cornelisse, C. J., Devilee, P., and Devlin, B. 2000. Mutations in *SDHD*, a mitochondrial complex II gene, in hereditary paraganglioma. Science 287: 848–851.

Baysal, B. E., Willett-Brozick, J. E., Lawrence, E. C., Drovdlic, C. M., Savul, S. A., McLeod, D. R., Yee, H. A., Brackmann, D. E., Slattery, W. H., III, Myers, E. N., Ferrell, R. E., and Rubinstein, W. S. 2002. Prevalence of *SDHB*, *SDHC*, and *SDHD* germline mutations in clinic patients with head and neck paragangliomas. J. Med. Genet. 39: 178–183.

Bourgeron, T., Chretien, D., Poggi-Bach, J., Doonan, S., Rabier, D., Letouze, P., Munnich, A., Rotig, A., Landrieu, P., and Rustin, P. 1994. Mutation of the fumarase gene in two siblings with progressive encephalopathy and fumarase deficiency. J. Clin. Invest. 93: 2514–2518.

Bourgeron, T., Rustin, P., Chretien, D., Birch-Machin, M., Bourgeois, M., Viegas-Pequignot, E., Munnich, A., and Rotig, A. 1995. Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency. Nat. Genet. 11: 144–149.

Brandon, M., Baldi, P., and Wallace, D. C. 2006. Mitochondrial mutations in cancer. Oncogene 25: 4647–4662.

Brouwers, F. M., Eisenhofer, G., Tao, J. J., Kant, J. A., Adams, K. T., Linehan, W. M., and Pacak, K. 2006. High frequency of *SDHB* germline mutations in patients with malignant catecholamine-producing paragangliomas: implications for genetic testing. J. Clin. Endocrinol. Metab. 91: 4505–4509.

Cecchini, G., Maklashina, E., Yankovskaya, V., Iverson, T. M., and Iwata, S. 2003. Variation in proton donor/acceptor pathways in succinate:quinone oxidoreductases. FEBS Lett. 545: 31–38.

Coller, H. A., Khrapko, K., Bodyak, N. D., Nekhaeva, E., Herrero-Jimenez, P., and Thilly, W. G. 2001. High frequency of homoplasmic mitochondrial DNA mutations in human tumors can be explained without selection. Nat. Genet. 28: 147–150.

Dahia, P. L., Ross, K. N., Wright, M. E., Hayashida, C. Y., Santagata, S., Barontini, M., Kung, A. L., Sanso, G., Powers, J. F., Tischler, A. S., Hodin, R., Heitritter, S., Moore, F., Dluhy, R., Sosa, J. A., Ocal, I. T., Benn, D. E., Marsh, D. J., Robinson, B. G., Schneider, K., Garber, J., Arum, S. M., Korbonits, M., Grossman, A., Pigny, P., Toledo, S. P., Nose, V., Li, C., and Stiles, C. D. 2005. A HIF1alpha regulatory loop links hypoxia and mitochondrial signals in pheochromocytomas. PLoS Genet. 1: 72–80.

Gallardo, M. E., Moreno-Loshuertos, R., Lopez, C., Casqueiro, M., Silva, J., Bonilla, F., Rodriguez, D. C., and Enriquez, J. A. 2006. m.6267G > A: A recurrent mutation in the human mitochondrial DNA that reduces cytochrome c oxidase activity and is associated with tumors. Hum. Mutat. 27: 575–582.

Gatenby, R. A. and Gillies, R. J. 2004. Why do cancers have high aerobic glycolysis? Nat. Rev. Cancer 4: 891–899.

Gottlieb, E. and Tomlinson, I. P. 2005. Mitochondrial tumour suppressors: A genetic and biochemical update. Nat. Rev. Cancer 5: 857–866.

Ishii, N., Fujii, M., Hartman, P. S., Tsuda, M., Yasuda, K., Senoo-Matsuda, N., Yanase, S., Ayusawa, D., and Suzuki, K. 1998. A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. Nature 394: 694–697.

Kaelin, W. G. 2005. Proline hydroxylation and gene expression. Annu. Rev. Biochem. 74: 115–128.

Kiuru, M. and Launonen, V. 2004. Hereditary leiomyomatosis and renal cell cancer (HLRCC). Curr. Mol. Med. 4: 869–875.

Kiuru, M., Lehtonen, R., Arola, J., Salovaara, R., Jarvinen, H., Aittomaki, K., Sjoberg, J., Visakorpi, T., Knuutila, S., Isola, J., Delahunt, B., Herva, R., Launonen, V., Karhu, A., and Aaltonen, L. A. 2002. Few FH mutations in sporadic counterparts of tumor types observed in hereditary leiomyomatosis and renal cell cancer families. Cancer Res. 62: 4554–4557.

Lee, S., Nakamura, E., Yang, H., Wei, W., Linggi, M. S., Sajan, M. P., Farese, R. V., Freeman, R. S., Carter, B. D., Kaelin, W. G., Jr., and Schlisio, S. 2005. Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. Cancer Cell 8: 155–167.

Neumann, H. P., Bausch, B., McWhinney, S. R., Bender, B. U., Gimm, O., Franke, G., Schipper, J., Klisch, J., Altehoefer, C., Zerres, K., Januszewicz, A., Eng, C., Smith, W. M., Munk, R., Manz, T., Glaesker, S., Apel, T. W., Treier, M., Reineke, M., Walz, M. K., Hoang-Vu, C., Brauckhoff, M., Klein-Franke, A., Klose, P., Schmidt, H., Maier-Woelfle, M., Peczkowska, M., Szmigielski, C., and Eng, C. 2002. Germ-line mutations in nonsyndromic pheochromocytoma. N. Engl. J. Med. 346: 1459–1466.

Neumann, H. P., Pawlu, C., Peczkowska, M., Bausch, B., McWhinney, S. R., Muresan, M., Buchta, M., Franke, G., Klisch, J., Bley, T. A., Hoegerle, S., Boedeker, C. C., Opocher, G., Schipper, J., Januszewicz, A., and Eng, C. 2004. Distinct clinical features of paraganglioma syndromes associated with *SDHB* and *SDHD* gene mutations. JAMA 292: 943–951.

Niemann, S. and Muller, U. 2000. Mutations in *SDHC* cause autosomal dominant paraganglioma, type 3. Nat. Genet. 26: 268–270.

Oyedotun, K. S., Sit, C. S., and Lemire, B. D. 2007. The Saccharomyces cerevisiae succinate dehydrogenase does not require heme for ubiquinone reduction. Biochim. Biophys. Acta 1767: 1436–1445.

Parker, W. H. 2007. Etiology, symptomatology, and diagnosis of uterine myomas. Fertil. Steril. 87: 725–736.

Piruat, J. I., Pintado, C. O., Ortega-Saenz, P., Roche, M., and Lopez-Barneo, J. 2004. The mitochondrial *SDHD* gene is required for early embryogenesis, and its partial deficiency results in persistent carotid body glomus cell activation with full responsiveness to hypoxia. Mol. Cell Biol. 24: 10933–10940.

Pollard, P. J., Briere, J. J., Alam, N. A., Barwell, J., Barclay, E., Wortham, N. C., Hunt, T., Mitchell, M., Olpin, S., Moat, S. J., Hargreaves, I. P., Heales, S. J., Chung, Y. L., Griffiths, J. R., Dalgleish, A., McGrath, J. A., Gleeson, M. J., Hodgson, S. V., Poulsom, R., Rustin, P., and Tomlinson, I. P. 2005. Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours which result from germline FH and SDH mutations. Hum. Mol. Genet. 14: 2231–2239.

Pollard, P. J., Spencer-Dene, B., Shukla, D., Howarth, K., Nye, E., El Bahrawy, M., Deheragoda, M., Joannou, M., McDonald, S., Martin, A., Igarashi, P., Varsani-Brown, S., Rosewell, I., Poulsom, R., Maxwell, P., Stamp, G. W., and Tomlinson, I. P. 2007. Targeted inactivation of fh1 causes proliferative renal cyst development and activation of the hypoxia pathway. Cancer Cell 11: 311–319.

Salas, A., Yao, Y. G., Macaulay, V., Vega, A., Carracedo, A., and Bandelt, H. J. 2005. A critical reassessment of the role of mitochondria in tumorigenesis. PLoS Med. 2: e296.

Saraste, M. 1999. Oxidative phosphorylation at the fin de siecle. Science 283: 1488–1493.

Schiavi, F., Boedeker, C. C., Bausch, B., Peczkowska, M., Gomez, C. F., Strassburg, T., Pawlu, C., Buchta, M., Salzmann, M., Hoffmann, M. M., Berlis, A., Brink, I., Cybulla, M., Muresan, M., Walter, M. A., Forrer, F., Valimaki, M., Kawecki, A., Szutkowski, Z., Schipper, J., Walz, M. K., Pigny, P., Bauters, C., Willet-Brozick, J. E., Baysal, B. E., Januszewicz, A., Eng, C., Opocher, G., and Neumann, H. P. 2005. Predictors and prevalence of paraganglioma syndrome associated with mutations of the *SDHC* gene. JAMA 294: 2057–2063.

Schofield, C. J. and Ratcliffe, P. J. 2004. Oxygen sensing by HIF hydroxylases. Nat. Rev. Mol. Cell Biol. 5: 343–354.

Selak, M. A., Armour, S. M., MacKenzie, E. D., Boulahbel, H., Watson, D. G., Mansfield, K. D., Pan, Y., Simon, M. C., Thompson, C. B., and Gottlieb, E. 2005. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell 7: 77–85.

Shambaugh, G. E., III. 1977. Urea biosynthesis I. The urea cycle and relationships to the citric acid cycle. Am. J. Clin. Nutr. 30: 2083–2087.

Smith, E. H., Janknecht, R., and Maher, L. J., III. 2007. Succinate inhibition of {alpha}-ketoglutarate-dependent enzymes in a yeast model of paraganglioma. Hum. Mol. Genet. 16: 3136–3148.

Sun, F., Huo, X., Zhai, Y., Wang, A., Xu, J., Su, D., Bartlam, M., and Rao, Z. 2005. Crystal structure of mitochondrial respiratory membrane protein complex II. Cell 121: 1043–1057.

Suzuki, T., Sato, M., Yoshida, T., and Tuboi, S. 1989. Rat liver mitochondrial and cytosolic fumarases with identical amino acid sequences are encoded from a single gene. J. Biol. Chem. 264: 2581–2586.

Szeto, S. S., Reinke, S. N., Sykes, B. D., and Lemire, B. D. 2007. Ubiquinone-binding site mutations in the Saccharomyces cerevisiae succinate dehydrogenase generate superoxide and lead to the accumulation of succinate. J. Biol. Chem. 282: 27518–27526.

Timmers, H. J., Kozupa, A., Eisenhofer, G., Raygada, M., Adams, K. T., Solis, D., Lenders, J. W., and Pacak, K. 2007. Clinical presentations, biochemical phenotypes, and genotype-phenotype correlations in patients with succinate dehydrogenase subunit B-associated pheochromocytomas and paragangliomas. J. Clin. Endocrinol. Metab. 92: 779–786.

Tomlinson, I. P., Alam, N. A., Rowan, A. J., Barclay, E., Jaeger, E. E., Kelsell, D., Leigh, I., Gorman, P., Lamlum, H., Rahman, S., Roylance, R. R., Olpin, S., Bevan, S., Barker, K., Hearle, N., Houlston, R. S., Kiuru, M., Lehtonen, R., Karhu, A., Vilkki, S., Laiho, P., Eklund, C., Vierimaa, O., Aittomaki, K., Hietala, M., Sistonen, P., Paetau, A., Salovaara, R., Herva, R., Launonen, V., and Aaltonen, L. A. 2002. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nat. Genet. 30: 406–410.

Tran, Q. M., Rothery, R. A., Maklashina, E., Cecchini, G., and Weiner, J. H. 2007. Escherichia coli succinate dehydrogenase variant lacking the heme b. Proc. Natl. Acad. Sci. USA 104: 18007–18012.

Vanharanta, S., Pollard, P. J., Lehtonen, H. J., Laiho, P., Sjoberg, J., Leminen, A., Aittomaki, K., Arola, J., Kruhoffer, M., Orntoft, T. F., Tomlinson, I. P., Kiuru, M., Arango, D., and Aaltonen, L. A. 2006. Distinct expression profile in fumarate-hydratase-deficient uterine fibroids. Hum. Mol. Genet. 15: 97–103.

Yankovskaya, V., Horsefield, R., Tornroth, S., Luna-Chavez, C., Miyoshi, H., Leger, C., Byrne, B., Cecchini, G., and Iwata, S. 2003. Architecture of succinate dehydrogenase and reactive oxygen species generation. Science 299: 700–704.

Van der Mey, A. G., Maaswinkel-Mooy, P. D., Cornelisse, C. J., Schmidt, P. H., and van de Kamp, J. J. 1989. Genomic imprinting in hereditary glomus tumours: evidence for new genetic theory. Lancet 2: 1291–1294.

Wallace, D. C. 2005. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu. Rev. Genet. 39: 359–407.

Warburg, O. 1956. On the origin of cancer cells. Science 123: 309–314.

Wei, M. H., Toure, O., Glenn, G. M., Pithukpakorn, M., Neckers, L., Stolle, C., Choyke, P., Grubb, R., Middelton, L., Turner, M. L., Walther, M. M., Merino, M. J., Zbar, B., Linehan, W. M., and Toro, J. R. 2006. Novel mutations in FH and expansion of the spectrum of phenotypes expressed in families with hereditary leiomyomatosis and renal cell cancer. J. Med. Genet. 43: 18–27.

Zanssen, S., and Schon, E. A. 2005. Mitochondrial DNA mutations in cancer. PLoS Medicine, 2: e401.

Mitochondria in Hematology

Stefanie Zanssen

The "mitochondrial clock" limits human life span by decreasing energy supply in the aged. If other mechanisms to limit human life span, such as severe infections or cancer, are eliminated, even the "healthy" aging rarely live beyond 100 years owing to mitochondrial degeneration, one of the three bases of aging. This refers to a substantial higher mutation rate of the mitochondrial genome than the nuclear genome (6×10^{-11} mutations per site per cell division), which is caused by a weaker mitochondrial repair system (Wallace 1999). The estimated mutation rate of the mitochondrial genome from pedigree-derived data, for somatic mutations, for substitution rates in cultured cells, and for stem cells is strikingly similar and corresponds to 1.10 mutations per site per million years (Taylor et al. 2003).

In fetal life the mutation load of the mitochondrial genome is very low, approximately 0.02%, but increases steadily over human life and aged people in their nineties may end up with 5–10% heteroplasmic mitochondrial DNA (mtDNA) mutations. If other mechanism of aging do not apply to a human being, mitochondrial degeneration seems to decrease energy production below the threshold level for living and typical "symptoms" of illness-free aging (Luft 1994; Murdock and Christiacos 2000) like atactic gait, mild tremor accompanying movements, or atrophy of skeletal muscles ensue. All these signs of aging can be objectified by morphologic correlations, e.g., by the finding of 10–15% of cytochrome c oxidase (COX) negative neurons in the immunostaining of the substantia nigra (personal observation) or by up to 2% of COX negative muscle fibers in aged people (Brierely et al. 1998; Muller-Hocker 1992).

The subclinical signs of aging aggravate to symptoms, which encompass seizures, encephalomyopathy, stroke like episodes and migraines, in patients with mitochondrial disease (DiMauro and Schon 2005). In contrast to a healthy fetus, a fetus with a mitochondrial disorder starts with a high mutation load of mtDNA, often inherited by his mother. Therefore tissue-specific threshold levels may be reached just after birth or in early childhood and devastating consequences for organs with high oxidative energy requirements, such as brain and muscle, will

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occur. Importantly in (neuro-) degeneration, it is not the presence of a mitochondrial mutation per se, but rather the inexorable and unregulated proliferation of mitochondria containing mutated mtDNAs, that leads to the devastating symptoms of patients. The phenomenon of accumulation of degenerated mitochondria with high mutation loads is best seen in muscle fibers of mitochondrial patients, where mitochondrial proliferation can be visualized as purplish blotches in "ragged-red fibers" (RRF). The factors that regulate mitochondrial turnover in postmitotic cells under physiological conditions and drive heteroplasmy to homoplasmy under stress conditions are not known at all. However, these postmitotic, energy-deficient muscle cells and neurons do not have any chance for self-renewal compared with dividing tissues.

Mitochondrial Haplotypes

Polymorphisms of mtDNA, derived over hundred thousands of years, have lead to the differentiation of approximately 15 mitochondrial haplogroups. These haplogroups characterize global ethnic groups (Elson et al. 2007). Even in the same ethnic group individuals differ in nucleotide positions of the mitochondrial genome, which specify their individual haplotype. Analysis of these mtDNA nucleotide variations reveals different categories of mutations. Most polymorphisms are neutral and do not alter the amino acid sequence of mitochondrial polypeptides. Adaptive polymorphisms may lead to amino acid changes in respiratory chain complexes, which might lead to functional changes for energy production and metabolism (Amo and Brandt 2007). Dependant on geographic regions, adaptive polymorphisms were subjected to positive selection and may have led to climate adaptation during migration waves of the ancestors of the "mitochondrial eve" from sub-Saharan regions to Europe or Asia. For example, mtDNA polymorphisms leading to slight uncoupling of the RC, which shifts ATP production towards heat production, might have been a benefit to sub-Saharan people when they migrated to Scandinavia. Polymorphisms may also influence longevity (Coscun et al. 2003; Santoro et al. 2006). On the other hand polymorphisms may be subjected to negative selection, if they lead to disease susceptibility. Pathogenic mutations occur and lead to mitochondrial disease, degeneration, and aging (Wallace 2005).

The mitochondrial genome contains several hotspot regions for mutations and polymorphisms (Wallace 2005). Regions with a low conservation index, like the mitochondrial control region, the D-loop, and especially its hypervariable regions, the ribosomal genes, as well as NADH dehydrogenase (ND) genes, contain most of the neutral polymorphisms. Adaptive polymorphisms are found in regions with an intermediate conservation index, including some ND genes but also include other mitochondrial polypeptides. Pathogenic mutations occur in highly conserved regions of the mitochondrial genome and have devastating functional consequences for RC function.

Aging of the Hematological System

The mitochondrial clock of aging runs on a different time scale for rapidly dividing tissues in comparison with postmitotic tissues. Postmitotic tissues, as specified earlier, expand a very low starting mutation load over decades, resulting in a somatic, tissue-specific haplotype, until when morphological signs of aging appear. A high frequency of mtDNA mutations in persons aged above 50 years (Wallace 1999) also affect normal human hematopoietic tissue (Gattermann et al. 1995; Shin et al. 2004). In contrast to postmitotic tissues, these mutations do not expand to levels, which would be detectable morphologically in the bone marrow or which would lead to an alteration of the bone marrow haplotype. Mitochondrial aging mutations of the hematological system are only detectable on the level of single cells. They might be erased on a short-term time base (Fig. 1) and mutations in hematologic cells seem to be strongly age dependant and may differ in different species (Shin et al. 2004). Mice show a strain specific and therefore genetic background specific pace of mitochondrial mutagenesis (Yao et al. 2007). On the other hand, if pathogenic mutations occur, energy deficient cells might be erased by negative selection from the bone marrow. In mitochondrial hematological disease (see Hematologic Mitochondrial Disorders), bone marrow cells with pathogenic mutations of mtDNA may be totally eliminated from the system and replaced by wild-type cells (Rotig et al. 1991).

Most of the somatic mutations introduced into hematologic cells occur on the single cell level and never change the individual haplotype. Approximately a quarter of analyzed CD34 single cells show additional mutations (mostly 1–2 mutations) to their inborn, germline mitochondrial haplotype in adults. In support of the mitochondrial aging theory, the fetal mutation rate for these single cells is much lower. Some early mutations are transmitted from their cell of origin, e.g., CD34 cells, to their granulocyte progeny (Shin et al. 2004). Most of these mutations are neutral and only few mutations target polypeptide-coding genes, alter conserved amino acids, and may have functional consequences.

Aging of Hematological Stem Cells

Evidence has been presented that hematopoietic stem cells (HSC) are also subject to aging (Geiger and van Zant 2002; Marley et al. 1999; Morrison et al. 1996), but may target HSC quality rather than quantity. The number of HSC and progenitor cells does not significantly decline in old age, probably because the quiescent HSC can undergo a carefully regulated process of self-renewal in order to maintain the stem cell pool (Dick and Lapidot 2005). The age-dependant mtDNA mutation frequency of single HSC in mice is with approximately 25% very similar to other human hematologic cells, but is not sufficient to alter the proliferation behavior of HSC (Yao et al. 2007). However, introduction of mtDNA mutations into HSCs may substantially contribute to the significant decrease of their functionality concerning

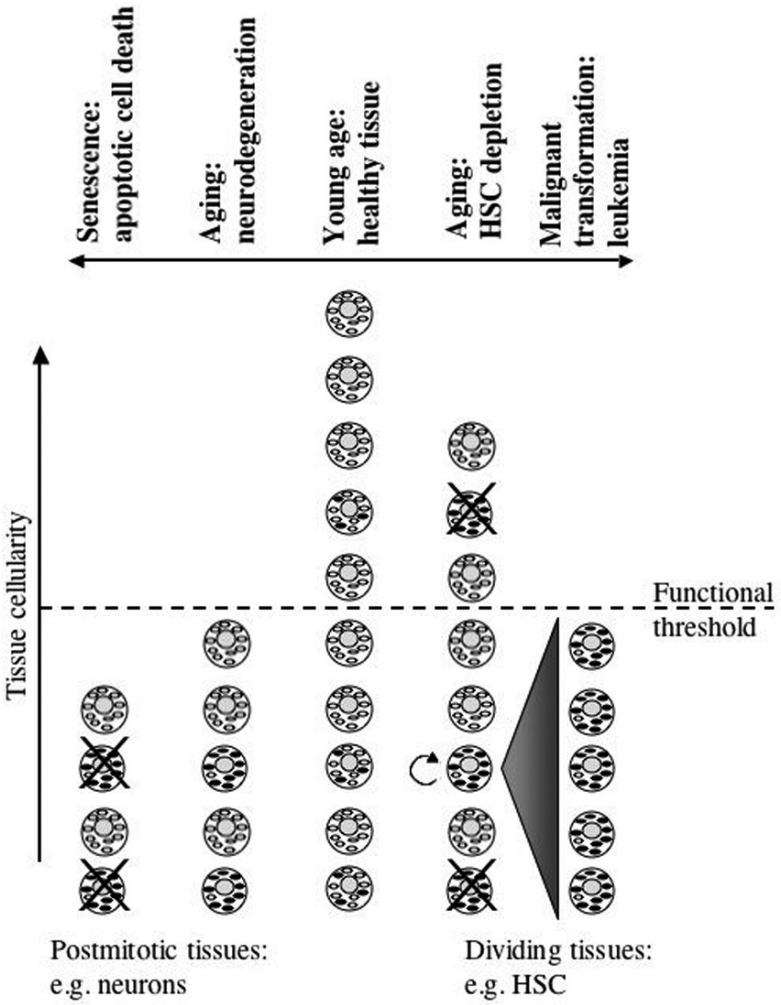

Fig. 1 Mitochondrial and cellular model of aging in postmitotic and dividing tissues. Crosses mark loss of cells over the life of an individual through mitochondrial-mediated cell death in postmitotic tissues and through negative selection in dividing tissues. The curved arrow represents oncogene activation leading to genetic instability of HSC with a high mitochondrial mutation load. The shaded triangle displays clonal evolution of transformed HSC. The minimum number of cells for normal tissue function is indicated by the dashed line

homing and lymphoid commitment with age (Liang et al. 2005; Morrisson et al. 1996). Furthermore, their differentiating progeny, being more dependent on mitochondrial energy production, may suffer much more functional impairment. Reported examples for a decreasing efficiency of hematopoiesis in combination with mtDNA mutations of the bone marrow are Pearson's Marrow Pancreas Syndrome (Rotig et al. 1990), sideroblastic anemia (Gattermann 2000), and myelodysplastic syndromes (Gattermann 2000; Linnartz et al. 2004) (see Hematologic Mitochondrial Disorders).

More investigations about mtDNA mutations and their functional consequences in HSCs are needed. However, it has been shown for other types of stem cells that morphologic signs of aging like cytochrome c oxidase (COX) negativity does not only occur in postmitotic tissues, but also in stem cells of rapidly dividing tissues. Furthermore, functional defects of mitochondria correspond to genetic changes. Lessons can be learned from elegant investigations of stem cells from colonic crypts as a model for the consequences of aging in HSCs and their progeny (Taylor et al. 2003). The clonal population of cells derived from single cell stem cells located at the base of colonic crypts has been hypothesized to harbor mtDNA mutations originally arisen in the stem cells itself. If these stem cell mtDNA mutations affect mitochondrial respiratory chain function, and if they exceed the threshold level for dysfunction (typically >80% mutated mtDNA) in their progeny, then one ought be able to observe both the dysfunction and the mtDNA mutation. Serial transverse sections from individual crypts of normal mucosa showed a marked age-related increase of COX deficient colonic crypts in the histochemical investigation. COX deficiency in colonic cells ranged from an average of ~1% in persons aged around 40 years, to an average of ~15% in old people aged around 80 years. PCR and mtDNA sequencing on the same crypts isolated by laser microdissection showed mtDNA mutations. These were mainly identified in COX-deficient crypts, and many, but not all, mutations were located in the three COX subunit genes encoded by mtDNA. Some mutations were also found in COX-positive crypts, but these were mainly neutral mutations or mutations in non-COX genes. Interestingly, there were some COX-deficient crypts in which no mtDNA mutations were found at all; presumably these crypts had mutations in nuclear encoded COX subunits or COX assembly genes. Altogether, there was a high mutational load in colonic stem cells. With the complete mitochondrial genome sequenced in 60 crypts, a total of 59 different mtDNA point mutations were detected. A significant number of mtDNA mutations were probably missed, because automated DNA sequencing has a detection limit of about 30% heteroplasmy.

The findings in stem cells and their progeny from colonic crypts support a role for mtDNA mutations in their ageing process. If a significant number of stem cells accumulate mutations, there will be a mixture of mutations within an individual, which may influence tissue function when impairing energy metabolism. Mutations found in HSCs or in stem cells from COX-deficient colonic crypts differ substantially from the spectrum seen in patients with mtDNA disease and may be so severe that they would be lethal in germline transmission.

The Drift to Homoplasmy in Aging Cells

Mitochondrial number and biogenesis must be adapted to external stimuli like, e.g., physical activity. The genes and signals that control directly the proliferation of mitochondria are unknown. Such signals may well be a part of the signal transduction pathway controlled by a "master regulator" gene that oversees many aspects of mitochondrial biogenesis—peroxisome proliferator-activated receptor gamma

coactivator 1α (PGC 1α) (Puigserver and Spiegelmann 2003)—but the details controlling the crosstalk between the nuclear and mitochondrial genome in proliferation are not known. MtDNA replication and mitochondrial division must also be synchronized to the cell cycle. The only so far known player in this process is cyclin D1 (Zanssen et al. 2004), a known oncogene in hematologic neoplasia (Donnellan and Chetty 1998). CCND1 plays an important role in cell cycle regulation, specifically in the G1-S phase of the cell cycle. When expressed during the cell cycle, it suppresses mitochondrial proliferation (Sakamaki et al. 2006).

During cell proliferation dividing cells may potentially donate a different complement of organelles and mitochondrial genomes to their progeny, a phenomenon called mitotic segregation. The phenotypic expression of cells vary in both space and time and become clinically important if an individual contains a heteroplasmic population of both wild-type and mutated mtDNAs. It is not an exaggeration to state that it is the unrelenting and uncontrolled accumulation of mutated mtDNAs, the drift to homplasmy for mutations in proliferating mitochondria, that lead to degeneration (DiMauro and Schon 2003). The accumulation of deficient mitochondria can also be observed in cancer, e.g., mitochondrial tumors like oncocytoma (Fig. 2).

Mechanisms

Selection

Active selection processes have been discussed to drive the mitochondrial mutation load towards homoplasmy in degenerating tissues. This drift to homoplasmy starts

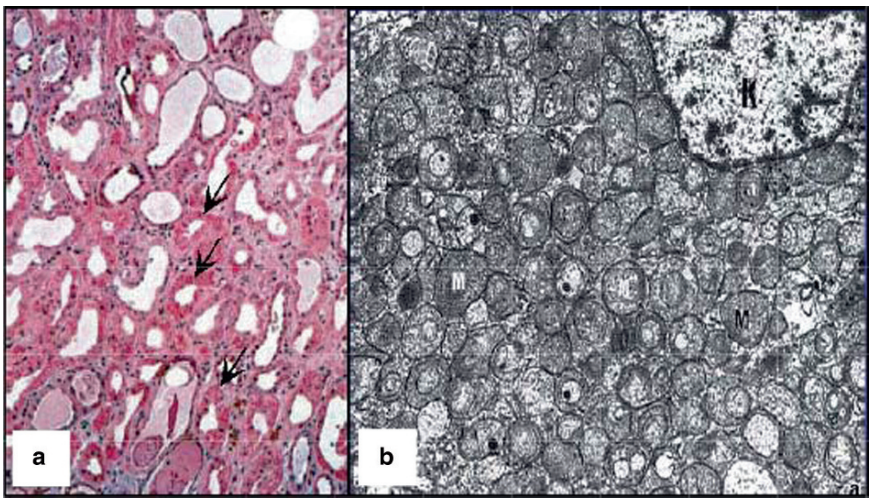

Fig. 2 Accumulation of deficient mitochondria in kidney oncocytoma. (**a**) H&E stain. Note large red-staining oncocytic cells in renal tubule cells (arrows). (**b**) EM of an oncocyte. The cytoplasm is packed with mitochondria (M). Nucleus (N) is at top right

on the intracellular, cytoplasmic level of all types of aging cells. In postmitotic cells, like, e.g., neurons of the substantia nigra, cells will undergo apoptotic degeneration after heteroplasmy exceeds the specific threshold level (Bender et al. 2006). Since these postmitotic cells are not renewable, symptoms like tremor may occur if a certain amount of cells is eliminated by this pathway. The same pathomechanism may apply to dividing cells like HSC. On the one hand, mitochondrial mutations will be more visible and detectable, because they do affect not only the stem cell of origin, but all their progeny (Shin et al. 2004). On the other hand, stem cells like HSC and their progeny will be eliminated after exceeding the bone marrow-specific threshold level. Since extinct HSC will be replaced by the quiescent HSC pool, symptoms of the aging bone marrow may be delayed in comparison with the aging CNS.

Positive selection, leading to the intracellular clonal expansion of mtDNA mutations, may be explained by "structural" and "functional" mechanisms (Kraytsberg et al. 2003). The "structural" mechanisms propose preferential proliferation of the mutant species based on properties of mutant DNA only, without referring to the function of any (mutant) tRNAs or proteins coded by mtDNA. For example, deleted mtDNA molecules were hypothesized, to replicate faster because they are shorter (Wallace 1989). Alternatively, deletion/inactivation of some negative regulatory sequences may lead to a replicative advantage of mutant mtDNA (Shoubridge et al. 1990). The "functional" mechanisms assume that the change in function of mutant tRNA/protein(s) is essential for expansion. For example, there may be preferential survival of dysfunctional mitochondria bearing mutant DNA, because decreased mitochondrial activity may reduce ROS-mediated damage, thereby retarding the removal of mutant organelles by mitochondrial turnover (de Grey 1997). Alternatively, there may be a positive feedback loop stimulating mtDNA synthesis in case of decreased ATP production (Chinnery et al. 1999; Hofhaus and Gattermann 1999; Shoubridge et al. 1990).

Random Processes

Besides selection mechanisms, random processes have been proposed to explain the expansion of mtDNA mutations in single stem cells and their progeny (Kraytsberg et al. 2003). The problem can be simulated with computers. A model of mtDNA replication in nondividing cells showed that marked intracellular drift can occur in postmitotic cells during a human life span. A single mutated mtDNA molecule may expand clonally to reach quasi homoplasmy in the absence of any replicative advantage (Elson et al. 2001).

Extensive computer modeling for proliferating tissues showed sufficient opportunity for a stem cell to achieve homoplasmy through unbiased mtDNA replication and sorting during cell division (Coller et al. 2001). Single cell analysis of mtDNA in healthy human epithelial tissue revealed that the model correctly predicts the considerable observed frequency of homoplasmic mutant cells (Nekhaeva et al. 2002). Therefore, random processes are sufficient to explain the incidence of

homoplasmic mtDNA mutations in aging stem cells. Recently, a model has been proposed for the accumulation of mtDNA mutations in ageing, cancer, and mitochondrial diseases which is also based on random genetic drift as the most important mechanism (Chinnery et al. 2002).

At the end however, independent on the mechanism leading to intracellular accumulation of mtDNA mutations in HSC, these cells and their progeny are subjected to strong negative selection if they harbor mutations in highly conserved amino acids or tRNAs with functional deteriorating consequences. Single HSC analysis shows that HSC and their progeny with such type of mutations are erased from the bone marrow in between month (Shin et al. 2004). There is only one event preventing the eradication of HSC with deficient mitochondria: the neoplasmic transformation of such cells.

Leukemogenesis

In 1956, the German biochemist and Nobel laureate Otto Warburg proposed that cancer is caused by an altered metabolism and by deranged energy processing in mitochondria (Warburg 1956). Biochemical studies showed that tumor cells have altered metabolic profiles and display high rates of glucose uptake and glycolysis, which originated from heritable injury of the mitochondria. Numerous studies carried out after Warburg's orginal observation have documented various deficiences of mitochondria in the cancer state. Nowadays it has been hypothesized that mitochondrial dysfunction may be linked to carcinogenesis via glycolytic, ROS-mediated, apoptotic or HIF-mediated pathways (Baysal 2003; Eng et al. 2003; Linnartz and Anglmayer 2004; Rustin and Rotig 2002).

Only the latter pathway has been proven so far (see chapter "Mitochondrial Tumor Supressors"). Mutations in the nuclear encoded mitochondrial enzymes SDH and fumarate hydrase (FH) have been shown to result in enzyme deficiencies in neoplasia. Mutations in SDH represent susceptibility to pheochromocytoma and paraganglioma and mutations in FH predispose to leiomyomatosis and renal cell carcinomas. SDH deficiency has been shown to turn on the HIF-mediated tumorigenic pathway (Selak et al. 2005), known to play a pivotal role for development of kidney cancers in von Hippel Lindau disease.

Mitochondrial Enzymes as Tumor Suppressors

A key conceptual question is now, if also mitochondrially-encoded complexes might function as tumor suppressors like SDH? MtDNA mutations like intragenic deletions (Horton et al. 1996), missense and chain-terminating point mutations (Polyak et al. 1998) and alterations of homopolymeric sequences (Habano and Nakamura 1998) are not only a characteristic of hematologic malignancies as described below, but of nearly every type of cancer cell. MtDNA mutations in tumors have been described not only in the hypervariable regions of the mitochondrial D-loop, the region

controlling mtDNA transcription and replication, and which is most prone to mutation, but also in all 22 tRNAs, both rRNAs, and all 13 of the mtDNA-encoded subunits of the respiratory chain complexes. The pattern of mutation distribution in cancer appears to follow almost exactly the evolutionary paths of mtDNA polymorphisms (Wallace 2005) and approximately 70% of mutations target nucleotide positions with a low conservation index specifying haplogroups and –types. However, less than 30% of mutations target conserved aminoacids of mitochondrially-encoded polypeptides or conserved regions of RNAs. Most of these mutations should cause such tremendous functional impairment, that looking at patient databases, they do not occur in mitochondrial patients.

Hematologic Findings in Mitochondrial Disorders

Pathogenic mutations of mtDNA cause a variety of disorders, and are classically encephalomyopathies. Clinical findings, including lactate acidosis, typically raise the diagnosis of a mitochondrial disease (DiMauro and Schon 2003). The first diagnostic step to support this diagnosis is the morphological analysis of a muscle biopsy. Mitochondria proliferate in tissues of patients and this is most strikingly seen in muscle, where mitochondrial proliferation can be visualized under the microscope as purplish blotches in "ragged-red fibers" (RRF) in the Gomorri trichrom stain. The RRF's contain the vast majority of mutated mtDNAs. Biochemically, mitochondrial diseases are characterized by respiratory chain deficiency and the analysis of respiratory chain (RC) complexes will give further support for a mitochondrial disease.

Finally, on the background of these diagnostic findings, molecular genetic analysis for mutations of the mitochondrial genome is performed to bring up the underlying gene defect. Not only muscle biopsies, which are invasive for patients, but also cultured fibroblasts from buccal mucosa swaps or leukocytes as well as platelets from blood serve as materials for gene analysis. Most mitochondrial disease do not encompass hematological symptoms, but are conveniently to diagnose on blood cells. This can be illustrated on the mitochondrial disease MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes), which is caused by the point mutation A3243G in mitochondrial transfer $RNA^{Leu(UUR)}$. The MELAS mutation impairs mitochondrial protein synthesis, thereby leading to RC dysfunction. MELAS patients do not show any hematological symptoms, but their leukocytes consistently display the mutation of the mitochondrial tRNA leucine gene. However, their heteroplasmy in blood is usually lower than in their skeletal muscle, e.g. 58% vs. 84% (Ciafaloni et al. 1992; Goto et al. 1991). In addition, the heteroplasmy level declines substantially with age in leucocytes (Poulton and Morton 1993). When paired blood DNA samples from patients with MELAS were investigated in 9–19 year intervals, the proportion of mutant mtDNA declined in all cases (range: 12–29%) (Rahman et al. 2001). On the other hand, the proportion of mutant mtDNA usually increases in affected tissues like skeletal muscle (Holme et al. 1995) and can be correlated to the progression of the disease.

These findings suggest a different fate for mutations of the mitochondrial genome in postmitotic and in dividing cells, which will be illustrated next on the hematological mitochondrial disorder Pearson's marrow pancreas syndrome.

Mitochondrial Hematologic Disorders

Pearson's Syndrome

Pearson's syndrome (PS) is a rare congenital disorder characterized by a severe sideroblastic anemia, exocrine pancreatic insufficiency, and other metabolic disturbances (Pearson et al. 1979). The anemia, which necessitates regular blood transfusions, is usually accompanied by neutropenia and thrombocytopenia. The bone marrow shows dysplastic changes, including moderate numbers of ringed sideroblasts and prominent vacuolization of precursor cells. Ringed sideroblasts are characterized by perinuclear accumulated mitochondria. Iron staining reveals that these mitochondria are overloaded with ferric iron (Fe^{3+}). PS is caused by large deletions of mtDNA (Roetig et al. 1990; Roetig et al. 1991). Severe complications of the agravating pancytopenia lead to death of most patients before the age of three. Interestingly, those who survive stop suffering from hematological symptoms, are able to eliminate the mutated clones from their blood system and become independent of blood transfusions. Favoring theories of different fates for mtDNA mutations in postmitotic and dividing tissues, there seems to be a selection against hematopoietic stem cells harboring a high percentage of deleted mtDNA molecules. With severe mtDNA defects like large deletions, a high level of heteroplasmy seems to be incompatible with accelerated stem cell proliferation, as required under conditions of hematopoietic stress. In such circumstances, selection pressure seems to be able to "purge" for energy deficient bone marrow cells, at least to a certain extent. If enough hematologic stem cells with wildtype mtDNA can be recruited to compensate for the degenerated, mutated ones, children can survive and live a life without any hematologic disturbances. Unfortunately, low rates of the same mutations in the central nerve system (CNS) of Pearson patients face the fate of mtDNA mutations in postmitotic tissues. After decades, surviving patients may develop the classical mitochondrial neuromuscular disorder Kearns Sayre Syndrome (Larsson et al. 1991; McShane et al. 1991; Nelson et al. 1992).

Acquired Idiopathic Sideroblastic Anemia

The clinical and morphological characteristics of the sideroblastic anemia found in children with PS are also found in the acquired idiopathic sideroblastic anemia (AISA) of adults. This points towards a same mitochondrial pathogenesis for both disease. AISA, a disorder included in the WHO classification of myelodysplastic syndromes (MDS) (Harris et al. 1999) under the name of "refractory anemia with

ringed sideroblasts" (RARS) is a clonal bone marrow disease arising from a multipotent hematopoietic stem cell (Gattermann 2000). It obtained its name from red cell precursors, the so-called ringed sideroblasts, which show the same abnormal iron deposits surrounding the cell nucleus as in PS.

In accordance to the theories of mitochondrial aging and carcinogenesis, sideroblastic anemia and MDS are disease of the aged. The incidence of RARS and other types of MDS is strongly age-dependent, with the majority of cases occurring after the age of 65. The crude annual incidence is about 1/100,000 (Aul and Gattermann 1992). In patients where morphological and functional changes in the bone marrow seem to be restricted to the erythropoietic lineage, the risk of leukemic transformation is very low. If granulopoietic and/or megakaryopoietic precursors also show dysplastic changes, the cumulative risk of leukemic transformation raises to 11% three years after diagnosis (Gattermann and Aul 1990; Germing et al. 2000).

Bone marrow morphology including iron staining confirm the diagnosis. Electronmicroscopically, iron granules correspond to mitochondria with massive iron accumulation in the mitochondrial matrix. Ringed sideroblasts may be accompanied by other dysplastic changes. Reversible causes of sideroblastic anemia like alcoholism, chronic lead poising, or antituberculosis treatment with isoniazide must be excluded. The diagnosis of a clonal bone marrow disease may be confirmed by detection of a clonal karyotype anomaly. There are no chromosomal abnormalities specifically associated with the sideroblastic phenotype, but chromosomal changes apparently provide the growth advantage to the clone harboring the mitochondrial defect (Gattermann 2000).

Patients' age makes the only curative approach, allogeneic stem cell transplantation, rarely feasible (Aul and Gattermann 1992; Gattermann 2000). Early detection, diagnosis and differentiation against other MDS subtypes may become feasible by use of mtDNA as a biomarker and may advance bone marrow transplantation and therefore prognosis (see chapter "Mitochondria and Cancer"). Supportive therapies include the hematopoietic growth factors erythropoietin and G-CSF, which can substantially reduce apoptosis of bone marrow cells, thereby improving blood counts. Since inefficient erythropoiesis causes increased intestinal iron absorption and secondary hemosiderosis, which is aggravated by blood transfusions, patients require iron chelation therapy.

Mitochondrial Iron Accumulation

Although the sideroblastic anemia of PS is caused by rearrangements of the mitochondrial genome, point mutations of mtDNA predominately in polypeptide encoding and ribosomal genes are found in the sideroblastic anemia of adults (Broeker et al. 1997; Gattermann et al. 1996; Gattermann et al. 1997; Gattermann 2000; Linnartz and Anglmayer 2004; Shin et al. 2003; Wang et al. 1999). In contrast to PS these mutations are not inherited but acquired in the bone marrow. Although mutations are present in low heteroplasmy, several mutations alter conserved sites and functional relevance has been demonstrated. Therefore, their potential is high to impair RC function and to be involved in the pathomechanism of iron accumulation.

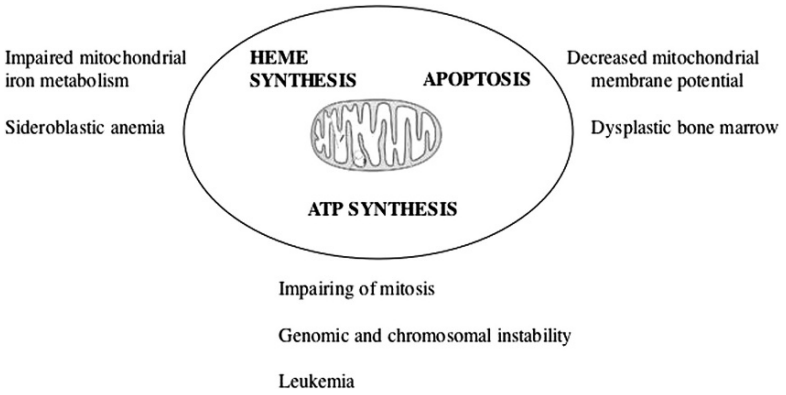

Fig. 3 Schematic representation of the pathogenesis of MDS and leukemogenesis

In erythropoietic cells, large amounts of iron are imported into mitochondria for heme synthesis (Gattermann et al. 1997) (Fig. 3). Iron must be provided in the chemical form of ferrous iron (Fe^{2+})[19], which can be exclusively incorporated by ferrochelatase into heme. In sideroblastic anemia, iron accumulates in the ferric form (Fe^{3+}). Iron overload appears to be attributable to a failure of RC complexes like NADH dehydrogenase, which may donate electrons to ferrochelatase to reduce ferric to ferrous iron. Hyperoxic conditions in sideroblasts may also participate in the pathogenesis. Uncoupling protein-2, which is expressed during erythroid differentiation, diminishes the proton gradient over the inner mitochondrial membrane, thereby stimulating RC activity to maintain the gradient. Accordingly, O_2 consumption is increased and a low-oxygen environment is created. A respiratory chain defect will slow down oxygen consumption and will thus create an hyperoxic environment in the mitochondrial matrix. If iron, which crosses the inner mitochondrial membrane in the ferrous form, becomes reoxidized ($\rightarrow Fe^{3+}$) by the Fenton reaction, it will be rejected by ferrochelatase and will thus accumulate in the mitochondrial matrix.

Myelodysplastic Syndromes

MDS are clonal myeloid disorders characterized by ineffective hematopoiesis and bone marrow cell dysplasia. Multiple combinations of lesions affect pluripotent hematopoietic progenitors and therefore affect myeloid, monocytic, erythroid and megakaryocytic lineages (Gattermann 2000). MDS are subdivided according to bone marrow cytology in 5 types by the FAB organization: refractory anemia (RA), two types of acquired sideroblastic anemia, refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEBt) and myelomonocytic leukemia (CMML) (Harris et al. 1999). Transformation resulting

in acute myeloid leukemia (AML) is the final stage in the multi-step process of MDS evolution (Crisan 2000).

The two types of AISA differ markedly with regard to survival and risk of leukemic transformation. If dysplastic changes on cytomorphology are confined to the erythroid lineage, the disorder is designated "refractory anemia with ringed sideroblasts" (RARS) and shows a low risk for leukemic transformation. If dysplastic changes affect granulocytopoiesis and/or megakaryopoiesis as well, the disorder is termed "refractory cytopenia with multilineage dysplasia and ringed sideroblasts" (RCMD-RS) and the risk for malignant transformation raises (Gattermann and Aul 1990; Germing et al. 2000). Patients with other types of MDS may also have ringed sideroblasts in their bone marrow, but to a lesser degree than $>15\%$ of nucleated red cell precursors. The basic pathomechanism of mitochondrial iron overload in MDS may be similar to sideroblastic anemia (see Aquired Idiopathic Sideroblastic Anemia).

Mutations of Mitochondrial DNA

The mitochondrial iron overload seems to be caused, as observed in the sideroblastic anemia of PS, by mutations of mitochondrial DNA. However, rearrangements of mtDNA, found in PS, could be excluded for MDS (Linnartz and Anglmayer 2004). Instead, mtDNA mutations have been identified in up to 50% of patients with MDS analyzed (Gattermann 2000; Linnartz and Anglmayer 2004; Schon and Zanssen 2005). The mutational spectrum of MDS patients encompassed half G to A transitions, and therefore represents oxidative damage (Beckmann and Koppenol 1996). Many mutations are found in polymorphic positions characterizing haplotype-specifying nucleotides. Haplotyping of a larger collection of patients with MDS and evolving leukemias suggested an overrepresentation of the haplotype U (personal observation), which has been suspected to be a mitochondrial cancer haplotype, especially seen in prostate cancer (Petros et al. 2005). The slow progression in MDS to acute leukemia over years allowed to monitor the shift of haplotype-specifying polymorphisms from "initial" homoplasmy of the germline haplotype to heteroplasmy to "final" homoplasmy of the tumor-specific haplotype. Interestingly, several MDS patients shifted from a germline haplotype U to the more common haplotype H during malignant transformation, which again raises the question, if certain mitochondrial germline haplotypes favor malignant transformation? Later on, the resulting tumor-specific haplotypes may favor adaption to tumor environment and growth (Zanssen and Schon 2005).

Besides polymorphisms, mutations in rRNA, tRNA and polypeptide encoding genes of the mitochondrial genome have been detected in patients with MDS (Gattermann 2000; Linnartz and Anglmayer 2004; Shin et al. 2003) and acute leukemias developing from MDS (Linnartz and Anglmayer 2004; Zanssen and Schon 2005). Most of these mutations may have the same functional relevance and pathogenic potential as the mtDNA rearrangements causing Pearson's syndrome. MtDNA mutations in the early types of MDS are characterized by a heteroplasmic, low mutation load. In the more advanced types of MDS, characterized by an increasing number of blasts in the bone

marrow, the mtDNA mutation load begins to increase steadily. During malignant transformation, when the blast content increases over 30% in the bone marrow, the mutational load of functional relevant mutations as well as polymorphisms increases dramatically. Finally, with a rate dependent on the myelopoietic cell line homoplasmy is reached in acute leukemia (Linnartz and Anglmayer 2004).

Apoptosis of the Bone Marrow

MDS is a representative apoptotic disease and the clinically characterizing pancytopenia as well as the typical morphologic dysplastic changes of the bone marrow are caused by premature apoptosis of bone marrow precursor cells. Apoptosis is significantly increased in the early MDS subtypes (Fig. 1, Fig. 3). Hyperactivation of the mitochondria-caspase-9 pathway was seen in low-risk MDS and appears to be more important for the induction of apoptosis than the death receptor-caspase-8 pathway (Tehranchi et al. 2001). Intrinsic defects within the clonal cells may be at least as important as extrinsic apoptosis-inducing signals. Mitochondria play a central role as regulators and effectors of apoptosis, and mitochondrial dysfunction can lead to necrotic and apoptotic cell death (Kroemer and Read 2000).

A number of drugs like chloramphenicol, a specific mitochondrial respiratory chain inhibitor, chronic lead poising, or antituberculosis treatment with isoniazide may produce sideroblastic anemia and secondary MDS by impairing mitochondrial function and inducing apoptosis (Wolvetang et al. 1994). Some MDS patients harbor mtDNA mutations in regions of mitochondrial ribosomal genes, which may enhance the vulnerability to antituberculotic drugs (Linnartz and Anglmayer 2004). Interestingly, they were treated with derivatives of these antibiotics against tuberculosis decades before they developed MDS. This raises the question, if mtDNA mutations predispose to RC impairment under certain antibiotic therapies.

Apoptosis and Degenerative Mitochondrial Haplotypes

Approximately 30% of patients with MDS and leukemia show polymorphisms specifying the neurodegenerative haplotype J (Linnartz and Anglmayer 2004), which are linked to another mitochondrial disorder: Leber's hereditary optic neuropathy (LHON) (Singh 1989). LHON is a bilateral subacute degeneration of the optic nerve leading to blindness (Chalmers and Schapira 1999) and is caused by hereditary missense mutations of the mitochondrial genome. Primary mutations are located at nucleotide positions 3460, 11778 and 14484 in genes encoding subunits of complex I of the respiratory chain (Howell et al. 1998). Very often, these mutations arise on the backbone of the so called neurodegenerative mitochondrial haplotype J, which harbors a couple of polymorphisms which are also seen as "secondary LHON mutations". These secondary mutations can not trigger LHON alone, but together and in synergy with one of the primary LHON mutations (Johns and Neufeld 1993). It has been suggested, that the degenerative changes in the optic nerve may be mediated

by apoptosis and that LHON mutations may predispose cells to apoptosis. In cybrid cell lines carrying the 3460 and 11778 LHON mutations a predisposition to Fas-induced apoptosis could be observed (Danielson et al. 2002). Furthermore, impairment in oxidative phosphorylation activity could be demonstrated in transmitochondrial cell lines bearing the LHON 11778 mutation (Hofhaus et al. 1996). In cell lines carrying the 3460 and 11778, but not the 14484 LHON mutation, a reduced respiration rate and an increased doubling time was found (Brown et al. 2000).

It is not clear, if mtDNA mutations with a high pathogenic potential, arisen on a degenerative mtDNA haplotype J may trigger premature apoptosis of hematopoietic cells (Zanssen and Buse 2003) comparable to apoptotic degeneration of the optic nerve in LHON. While apoptosis is significantly increased in "early" MDS types, it is comparable to normal controls in advanced MDS (Berger et al. 2001, Parker et al. 1998). Early MDS types start with a low, heteroplasmic mutation load, which may induce apoptosis of bone marrow precursors following a pathway similar to LHON.

When MDS types advance, the mtDNA mutation load increases dramatically in a cell line specific manner. With more severe mitochondrial dysfunction a severe ATP-deficit may occur, favoring chromosomal lesions which help to escape apoptotic control and drive neoplastic transformation to acute leukemia. Bone marrow cells of a patient with LHON and malignant lymphoma, carrying a homoplasmic primary mutation, were not sensitized to apoptotic death or to cytotoxic drugs in a detrimental manner (Nikoselainen 1992) after treatment with combination chemotherapy (Zanssen and Buse 2003). A mitochondrial gene dosage-dependent fate of the cell for degeneration or carcinogenesis has also been suggested for hereditary mitochondrial cancers (see chapter "Mitochondrial Tumor Supressors") (Eng et al. 2003).

ATP Deficiency and Chromosomal Instability

In mammalian cells a hierarchy of ATP-consuming processes is found. If energy supply becomes inadequate, cells shut down their metabolic activities in a certain order (Fig. 3). Pathways of macromolecule synthesis, i.e. protein and RNA/DNA synthesis, are most sensitive to energy supply (Buttgereit and Brand 1995). This may be pertinent to the proliferation and function of bone marrow cells in MDS. In a study of ineffective erythropoiesis in sideroblastic anemia, an accumulation of early polychromatic cells in G2 and the presence of cells which were apparently arrested after a period in DNA synthesis (Wickramasinghe and Chalmers 1968) could be demonstrated. DNA synthesis was rarely seen in cells with pronounced iron deposits. Electron microscopy studies corroborated the concept that cells with large quantities of mitochondrial iron deposits become arrested in their progress through the cell cycle (Wickramasinghe and Hughes 1978). This study also revealed that the accumulation of increasing quantities of iron-containing material within the mitochondria of an erythroblast was frequently associated with a depression of RNA synthesis and a more substantial depression of protein synthesis. Such

impairment of macromolecule synthesis may partly explain the dysplastic phenotype of bone marrow cells in MDS.

Missense mutations in mitochondrial encoded ATPase genes have been reported in MDS (Gattermann 2000; Linnartz and Anglmayer 2004; Shin et al. 2003) as well as in ovarian carcinomas (Liu et al. 2001). Furthermore, many of the mutations described in MDS may have the functional potential to impair energy production, when they become homoplasmic. The resulting ATP deficiency in dividing cells may represent a major contributor to chromosomal and genomic instability. This may be examplified on MDS an its evolving acute leukemias. Chromosomal instability seems to be virtually absent in patients with purely erythroid sideroblastic anemia, who display only mild RC defects caused by low heteroplasmy (Broker et al. 1998). These patients have the lowest frequency of karyotype anomalies, the lowest incidence of leukemic transformation, and the best survival among all MDS patients (Gattermann and Aul 1990). Karyotype anomalies in other subtypes of MDS are characterized by losses or gains of whole chromosomes as well as deletions of chromosome arms. Specific translocations occur during malignant transformation towards acute leukemia. In advanced subtypes of MDS, characterized by a steady increase of the mitochondrial mutation load, the threshold level under which apoptotic mechanisms predominate may be overcome. A rising ATP deficit of cells will lead to difficulties in correctly segregating their chromosomes during mitosis, since the mitotic spindle apparatus depends on ATP-consuming motor proteins. Furthermore, ATP-dependent DNA repair mechanisms may fail and contribute to genomic instability. The next step would result in malignant transformation and produce the evolving acute leukemias in MDS. On the one hand, insertion of mitochondrial COX mutations found in MDS into the rho^0 cell system showed that they do not impair energy metabolism severely (Meunier et al. 1998). On the other hand, typical chromosomal rearrangements of tumors including hematologic malignancies can be introduced into rho^0 cells, which are completely depleted for their mtDNA (Singh et al. 2005). More functional studies are under urgent need to investigate the relation between ATP deciciency and genomic instability.

Mitochondrial Myopathy, Lactic Acidosis, and Sideroblastic Anemia

Mitochondrial myopathy, lactic acidosis and sideroblastic anemia (MLASA or MSA), is a rare disease with autosomal recessive inheritance. The hallmark features include progessive exercise intolerance during childhood aggravating to mitochondrial myopathy, onset of sideroblastic anemia around adolescence and basal lactic acidosis (Casas and Fischel-Ghodsian 2004; Inbal et al. 1995; Rawles and Weller 1974). Homozygous missense mutation in the pseudouridine synthase 1 gene (PUS1), which maps to chromosome 12q24.33, have been found in all patients with MLASA from two families (Casas et al. 2004). The resulting amino acid change affects a highly conserved amino acid, and appears to be in the catalytic center of the protein, PUS1p. A mitochondrial

targeting sequence suggests a mitochondrial location for the enzyme. Deficient pseudouridylation of mitochondrial tRNAs anticodons may alter their wobble capacity and may slow down mitochondrial protein synthesis. The activity of respiratory chain complexes with mitochondrially-encoded subunits is decreased, whereas succinate dehydrogenase and citrate synthase reference enzyme activities were increased. Therefore MLASA seems to be a mitochondrial disorder on the translational level. As in PS and RARS, erythropoiesis turns out to be particularly vulnerable to mitochondrial dysfunction. This is not surprising since heme synthesis, the major metabolic task of erythroblasts, is heavily dependent on mitochondrial activity.

Mitochondria in Hematologic Malignancies

Leukemia

The first investigations on mitochondria in leukemia decades ago started on the morphological level. Electron microscopy showed alterations of the mitochondrial network: in myeloblastic leukemia the mitochondria are less numerous and smaller than in normal cells at the same stage of maturation (Bessis 1973; Schumacher et al. 1974). Furthermore circular dimers and complex catenated forms of mtDNA were first observed in acute leukemia (Clayton and Smith 1975; Clayton and Vinograd 1967; Clayton and Vinograd 1969), and later on confirmed by gross abnormal dimer formation using molecular techniques for MDS (Hatfill and Kirby 1994). The latter observations may be linked to the unusual modus of mtDNA replication, since its two strands are replicated from two distinct origins of replication. DNA synthesis proceeds asymmetrically until it is completed on both strands, resulting in a catenated pair of circles. The linked rings are broken and resealed to produce two daughter duplex mtDNAs. The whole process takes a relatively long time – about 2 h. Under circumstances of continuous proliferative stress, mtDNA replication is increased, but the process of breaking and resealing daughter strands of mtDNA, may not be able to accelerate to the same extent, thus leaving a significant proportion of replicated circular mtDNA strands interlinked. This energy-dependant process may fail because mitochondria become energy deficient and may be enhanced by a vicious circle, since complex forms of mtDNA are probably unsuitable for normal RNA synthesis.

Despite the downregulation of mitochondrial biogenesis observed in many tumors including leukemia, mitochondrial DNA is consistently amplified (2- to 50-fold) in acute leukemia evolving from MDS (personal observation), in blast cells of acute myeloid leukemia (AML) (Gattermann and Aul 1997), as well as in chronic granulocytic leukemia (CGL) during transformation from chronic phase (Boultwood et al. 1996). In about half the patients, the increase in the mtDNA/nuclear DNA ratio was more than 8-fold. Deletions and duplications or multiple deletions are not found in leukemia (personal observation).

Mutations of Mitochondrial DNA

Several laboratories have reported unexpected large numbers of somatic mutations in leukemia. Investigations of the mitochondrial control region revealed mtDNA mutations in acute lymphoblastic leukemia (ALL) (Ivanova et al. 1998), chronic lymphocytic leukemia (CLL) (Carew et al. 2003) and AML (Grist et al. 2004). Some of these studies compared mtDNA from clonal bone marrow disorders with mtDNA from healthy individuals (Ivanova et al. 1998; Shin et al. 2003), and produced ambiguous results. Sequence changes between patients and controls may simply reflect ethnic-specific haplotype variation, and clonal hematopoiesis cannot be compared with a healthy polyclonal bone marrow. In a clonal marrow, the somatic mitochondrial haplotype of a particular stem cell is amplified, whereas a healthy control sample contains mtDNA from many different stem cells.

Whole mitochondrial genome scans allowed a more precise view. Approximately 40% of patients with acute and chronic leukemias (He et al. 2003) and up to 50% of patients with acute leukemias developing from MDS (Linnartz and Anglmayer 2004) show somatic point mutations of mitochondrial DNA. Several point mutations of mtDNA may accumulate in the bone marrow of one patient and may act synergistically. In MDS the segregation behavior of mtDNA mutations can be studied during disease progression towards leukemia, since defined time and stage intervals characterized by morphological parameters are available. Heteroplasmic point mutations increase over RAEB towards RAEBt and increase dramatically and rapidly towards homoplasmy during malignant transformation to AML. This increasing mtDNA mutation load is not only stage and therefore time-specific, but also dependant on the myelopoietic cell line. Since most of mtDNA mutations found in leukemia never have been described in healthy individuals, they may have significant functional relevance on their way to homoplasmy.

In patients with adult-onset ALL (He et al. 2003) could be verified, that aging mutations only become apparent as neoplasia-specific mitochondrial haplotypes if they occur in a HSC which further on undergoes malignant transformation and forms a clonal bone marrow (Fig. 1). When an ALL patient was treated with chemotherapy, the malignant clone harboring the mtDNA mutation was strongly suppressed and patient's germline haplotype was reconstituted in the bone marrow. However, advanced techniques identified a persisting mutation load of 0.1% in chemotherapy-induced remission and suggested that the malignant clone resisted. The neoplasia-specific haplotype dominated the bone marrow again, when the patient relapsed later on. Similar results were obtained for a patient with AML (Yao et al. 2007).

Lymphoma

Circular dimers of mtDNA were not only observed in AML but also in poorly differentiated lymphocytic lymphoma (Firkin and Clark-Walker 1979). A marked

shift from the predominant circular monomeric form to circles with twice the normal circumference, as well as concatenated forms of mtDNA were reported. However, there is no clear correlation between the neoplastic state of cells and the occurrence of complex forms of mtDNA, since the latter have also been observed in human B cells transformed by Epstein–Barr Virus or phytohemagglutinin (PHA) (Miguel et al. 1982). At a lower frequency, complex forms of mtDNA were also found in lymphocytes isolated from donor blood (Christiansen and Christiansen 1983).

Mutations of Mitochondrial DNA

Only one study recently investigated the relationship between mtDNA mutations, reactive oxygen species (ROS) generation, and clinical outcomes in CLL patients (Carew et al. 2003). This study focused on 6 regions, the typical hotspot positions of the D-loop, the low conservation index regions of NADH dehydrogenase (ND) and cytochrome b genes and two high conservation index regions (COXII and ATPase6/8). The rate of CLL patients with mtDNA mutations in this study is higher than in MDS or AML and reaches 100%.

Two groups of CLL patients, one characterized by moderate and the other by advanced disease stage necessitating chemotherapy, accumulated nearly the same number mtDNA polymorphisms and mutations in their CLL cells. Interestingly, the number of mutations leading to amino acid substitutions with supposed functional relevance is more than 3-fold higher in the advanced stage, chemotreated cases than in the group with moderate disease.

Functional Impact and Chemoresistance

Furthermore, patients from the advanced disease group, who were refractory to conventional therapeutic agents tended to have higher mutation rates than patients who responded to treatment. This observation may have two reasons. On the one hand, chemotherapy with certain DNA-damaging agents may cause additional mtDNA mutations. On the other hand, a comparison of the number of mutations before and after chemotherapy suggests that patients harbored these mutations even before antineoplastic therapy was started.

Therefore, a high number of functional relevant mtDNA mutations resulting in mitochondrial deficiency may significantly contribute to tumor growth and chemoresistance. Mitochondria generated ROS were suggested as a primary pathway leading to tumorigenesis (Eng et al. 2003) (see chapter “Oxidative Phosphorylation: the Link between Metabolism and Cancer) and a ROS mediated mechanism has been suggested for CLL (Carew et al. 2003). The increased ROS production could lead to additional mutations in both nuclear DNA and mtDNA, contributing to genetic instability and disease progression. Because mitochondria play an essential role in apoptosis, the increase in ROS may also affect the sensitivity of cancer cells to chemotherapeutic agents. Previous studies indicated that ROS can stimulate

cell proliferation and activate prosurvival transcription factors such as NF-κB (Haddad 2002; Liu et al. 2002). Taking into consideration that many antiapoptotic proteins such as XIAP are under the transcriptional control of NF-κB, it is plausible that this effect could contribute to drug resistance and disease progression.

Potential Pathways of Mitochondrial Leukemogenesis

Lessons, how mtDNA mutations in MDS and hematologic malignanices may contribute substantially to carcinogenesis may be learned by applying the SDH deficiency model for carcinogenesis to other RC complexes. As reported above, aging mutations arise and are erased in between month (Shin et al. 2003) (Fig. 1). The only chance for somatic mtDNA mutations to alter the haplotype in tumors and to produce deficiencies of complex I, III, or IV is their occurrence in a stem cell, which further undergoes malignant transformation. Investigations on the single cell level prove this in showing that the number of "aging haplotypes" of a normal bone marrow (with highest rates for differentiated cells like granulocytes) is significantly higher than the number of haplotypes of a clonal, leukemic bone marrow (Yao et al. 2007).

It is unknown, if this is a coincidence, or if these aging mutations contribute to the process of malignant transformation. Silent mutations, identified in solid tumors or clonal bone marrow disorders, are not supposed to play an important general role in carcinogenesis or leukemogenesis (He et al. 2003). However, newly arising polymorphisms include besides silent mutations mtDNA mutations, which alter mildly conserved amino acids in RC complexes. When these simultaneously arising mutations convert patients germline haplotype into a tumor specific haplotype, a selective advantage due to the new haplotype may result (Zanssen and Schon 2005; Petros et al. 2005). Comparable to adaptive polymorphisms, which gave selective advantages like climate adaption during migration waves of our ancestors, polymorphisms in neoplasia may adapt tumors for example to their hypoxic environment, angiogenic insufficiency or metabolic shift.

On the other hand, mutations at well-conserved sites of mtDNA with a functional relevance (Meunier et al. 1998), which are supposed to be even more devastating than mutations found in mitochondrial disorders, may be involved in carcino- and leukemogenesis or at least in tumor aggressiveness. The latter is supported by findings of a 3 fold higher number of mtDNA mutations with functional relevance in patients with advanced stage chronic lymphatic leukemia (CLL) than in patients with moderate CLL. Furthermore, a high number of mutations in advanced CLL increases the chance for chemoresistance (Carew et al. 2003).

Cell's fate for degeneration or for malignant transformation dependant on the mutation load seems also to apply to both, deficiency of nuclear and mitochondrial encoded polypeptides of the RC and suggests a common pathway of tumorigenesis. SDH-deficiency, where homozygote SDH mutations cause neurodegeneration, whereas heterozygote mutations trigger carcinogenesis (Eng et al. 2003) may find

an analog in MDS. In heteroplasmy the apoptotic degeneration of the bone marrow predominates the clinical picture, whereas homoplasmy is reached when MDS transforms to AML (Linnartz and Anglmayer 2004). It remains open so far, if other RC complexes than SDH, for example complex I, very often mutated in tumors, may also impair SDH and Krebs cycle function and lead to succinate accumulations, which furtheron turn on the HIF-mediated tumorigenic pathway (Fig. 4).

New pathways of mitochondrial carcinogenesis, different from the HIF-mediated one, are coming into play. Studies of non-hereditary mitochondrial tumors like oncocytoma (see chapter "Mitochondria and Oncocytomas") reveal oncogenes, which are capable to induce degeneration and carcinogenesis. Oncocytic cells of the kidney show both: morphological signs of uncontrolled proliferation of degenerated mitochondria as well as tumorous growth (Fig. 2). A subgroup of kidney oncocytoma with aberrations of chromosome 11q13 turn on expression of the regional oncogene CCND1 (Zanssen et al. 2004), which besides its role in driving cell division is a negative regulator of mitochondrial proliferation and biogenesis (Sakamaki 2006).

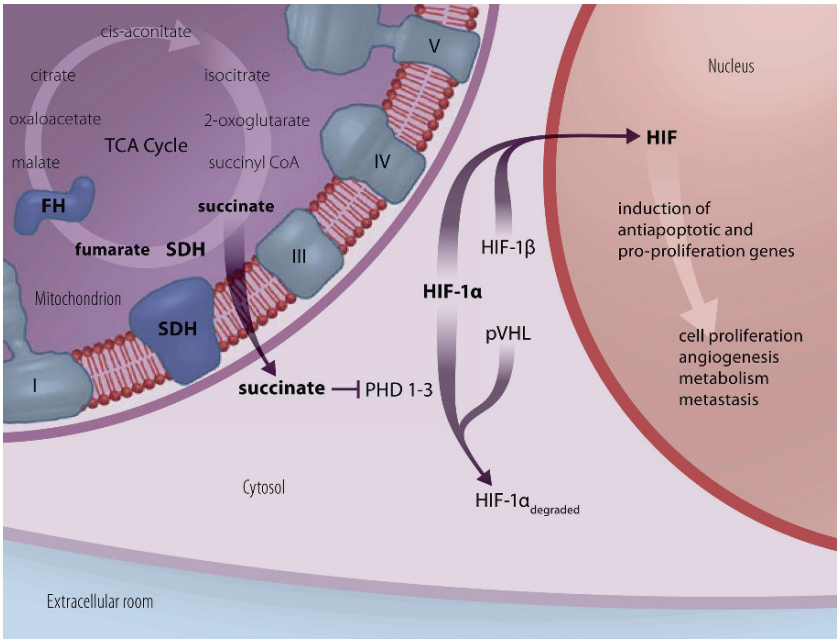

Fig. 4 Schematic model for tumorigenesis depending on the succinate signaling pathway. Owing to SDH inhibition succinate accumulates intramitochondrially and is transported into the cytosol. Here, its high concentration inhibits the prolyl hydroxylases and thereby HIF-1α hydroxylation and degradation. HIF is now elevated and induces transcription of genes involved in tumor progression

Early Detection of Hematologic Malignancies

Mitochondrial genomic alterations do not characterize only hematological malignancies but also solid tumors (Penta et al. 2001). In patients with breast or prostate cancer, which have a high mortality rate, it has been shown that mtDNA mutations occur in 70% and 95% of cases, respectively (Jeronimo et al. 2001). Due to their clonal nature and high copy number, mitochondrial mutations may represent a powerful molecular marker for noninvasive detection of MDS and hematologic malignancies.

The pattern of mutation distribution over the mitochondrial genome in hematologic malignancies and cancer appears to follow almost exactly the evolutionary paths of mtDNA polymorphisms (Wallace 2005). Analysis of mtDNA nucleotide variation in evolution reveals different categories of mutations: most are neutral and/or adaptive polymorphisms, few are pathogenic. MtDNA polymorphisms, which normally arise over the course of thousands of years, accumulate in hematologic malignancies and tumors within a few weeks. It is unclear at present by which mechanisms mtDNA mutations are fixed in hematologic neoplasia (see "Mitochondrial Enzymes as Tumor Suppressors"). Predominantly, mtDNA mutations occur in 3 primary mtDNA "hotspot" positions in the mitochondrial D-loop in approximately one third of cancer patients, and in some cancers, such as gastric cancer or lung carcinoma, they occur in approximately 70% of patients. Seven other nucleotide positions in the mitochondrial control region define a second hotspot mutated in approximately another third of patients. In conclusion, the probability of detecting a cancer-associated mutation in the D-loop of patients with MDS and hematologic malignancies is very high (approximately 1 in 20 bases). MtDNA mutations occur less often in the coding region of the mitochondrial genome, especially in the conserved protein-coding genes. The detection frequency is much lower in this area (approximately 1 in 500 bases).

Therefore, analysis of the "hotspot regions" via sequencing or oligo array chips represents a powerful tool for early detection of MDS, hematologic malignancies, and tumors (Modica-Napolitano and Kulawiec 2007; Verma et al. 2007) (see also chapter "Mitochondria and Cancer"). In MDS, on the one hand single hotspot mutations in very low heteroplasmy may point towards the subtype refractory anemia with ring sideroblasts (RARS). The good prognosis and benign character of this subtype would implicate clinical surveillance and if indicated, hematologic supportive therapy. On the other hand, several hotspot mutations with intermediate heteroplasmy may suggest a development towards refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS). The risk for progression towards leukemia is now much higher and if patients age allows this, the planning of a bone marrow transplantation should begin immediately.

Beyond early cancer detection, mtDNA may serve as a biomarker for staging of known leukemias and lymphomas. Heteroplasmy as well as the number of mutations per tumor seem to increase in advanced stages (Carew et al. 2003; Linnartz and Anglmayer 2004). A declining heteroplasmy under chemotherapy reflects an

efficient elimination of the malignant clone and may be used for therapy monitoring. On the other hand, an increase of heteroplasmy after chemotherapy may indicate chemoresistance of the malignant clone.

The biomarker mtDNA may also serve for susceptibility and predisposition screening. Recent research suggests that some polymorphisms in specific mtDNA genes may predispose to breast cancer (e.g. in ND3) (Canter et al. 2003), prostate cancer (e.g. in COX genes) (Petros et al. 2006), and MDS (personal observation). Interestingly, several COX polymorphisms characterize African mitochondrial haplotypes, and African-Americans have a substantial higher rate of prostate cancer than do Europeans. Also, germline European mitochondrial haplotypes defined by COX variants double the risk for prostate and several other cancers. Hence, addition of cancer haplotype-defining polymorphisms to the "mikromitochip" may give the chance to identify high risk individuals and families, since mtDNA is inherited maternally, for certain cancers and for cancer prevention.

References

Amo T, Brandt MD. Were effecient mitochondrial haplogroups selected during migration of modern humans? A test using modular kinetic analysis of coupling in mitochondria from cybrid cell lines. Biochem J 2007;404:345–351.

Aul C, Gattermann N, Schneider W. Age-related incidence and other epidemiological aspects of myelodysplastic syndromes. Br J Haematol 1992;82:358–367.

Baysal BE. On the association of succinate dehydrogenase mutations with hereditary paraganglioma. Trends Endocrinol Metab 2003;14:453–459.

Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrende EC, Myssiorek D, Bosch A, van der Mey A, Taschner PE, Rubinatein WS, Myers BN, Richard CW 3rd, Corne Lisse CJ, Devi Lee P, Devlin B. Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. Science 2000;287:848–851.

Beckman JS, and Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 1996;271:C1424–1437.

Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, Jaros E, Hersheson JS, Betts J, Klopstock T, Taylor RW, Turnbull DM. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet 2006;38(5):515–517.

Berger G, Hunault-Berger M, Rachieru P, Fontenay-Roupie M, Baranger L, Ifrah N, Zandecki M. Increased apoptosis in mononucleated cells but not CD34+ cells in blastic forms of myelodysplastic syndromes. The Hematol J 2001;2:87–96.

Bessis M. Living blood cells and their ultrastructure. 1973. Berlin: Springer.

Boultwood J, Fidler C, Mills KI, Frodsham PM, Kusec R, Gaiger A, Gale RE, Linch DC, Littlewood TJ, Moss PAH, Wainscoat JS. Amplification of mitochondrial DNA in acute myeloid leukaemia. Br J Haematol 1996;95:426–431.

Brierley EJ, Johnson MA, Lightowlers RN, James OFW, Turnbull DM. Role of mitochondrial DNA mutations in human aging: implications for the central nervous system and muscle. Ann Neurol 1998;43:217–223.

Bröker S, Meunier B, Rich P, Gattermann N, Hofhaus G. mtDNA mutations associated with sideroblastic anaemia cause a defect of mitochondrial cytochrome c oxidase. Eur J Biochem 1998;258:132–138.

Brown MD, Trounce IA, Jun AS, Allen JC, Wallace DC. Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778, or 14484 Leber's hereditary optic neuropathy mitochondrial DNA mutation. J Biol Chem 2000;275:39831–39836.

Buttgereit F, Brand MD. A hierarchy of ATP-consuming processes in mammalian cells. Biochem J 1995;312:163–167.

Canter JA, Kallianpur AR, Parl FF, Millikan RC. Mitochondrial DNA G10398A polymorphism and invasive breast cancer in African-American women. Cancer Res 2005;65(17):8028–8033.

Carew JS, Zhou Y, Alibar M, Carew JD, Keating MJ, Huang P. Mitochondrial DNA mutations in primary leukemia cells after chemotherapy: clinical significance and therapeutic implications. Leukemia 2003;17:1437–1447.

Casas KA, Bykhovskaya Y, Mengesha E, Wang D, Yang H, Taylor K, Inbal A, Fischel-Ghodsian N. Gene responsible for mitochondrial myopathy and sideroblastic anemia (MSA) maps to chromosome 12q24.33. Am J Med Genet 2004;127A:44–49.

Casas KA, Fischel-Ghodsian N. Mitochondrial myopathy and sideroblastic anemia. Am J Med Genet 2004;125A:201–204.

Chalmers RM and Schapira AH. Clinical, biochemical and molecular genetic features of Leber's hereditary optic neuropathy. Biochim Biophys Acta 1999;1410:147–158.

Chinnery PF, Samuels DC. Relaxed replication of mtDNA: a model with implications for the expression of disease. Am J Hum Genet 1999;64:1158–1165.

Chinnery PF, Samuels DC, Elson JL, Turnbull DM. Accumulation of mitochondrial DNA mutations in ageing, cancer, and mitochondrial disease: is there a common mechanism? Lancet 2002;360:1323–1325.

Christiansen G, Christiansen C, Zeuthen J. Complex forms of mitochondrial DNA in human B cells transformed by Epstein-Barr virus. J Cancer Res Clin Oncol 1983;105:13–19.

Ciafaloni E, Ricci E, Shanske S, Moraes CT, Silvestri G, Hirano M, Simonetti S, Angelini C, Donati A, Garcia C, Martinuzzi A, Mosewich R, Servidei S, Zammarchi E, Bonilla E, DeVivo DC, Rowland LP, Schon EA, DiMauro S. MELAS: clinical features, biochemistry, and molecular genetics. Ann Neurol 1992;31:391–398.

Clayton DA, Smith CA. Complex mitochondrial DNA. Int Rev Exp Pathol 1975;14:1–67.

Clayton DA, Vinograd J. Circular dimer and catenate forms of mitochondrial DNA in human leukaemic leukocytes. Nature 1967;216:652–657.

Clayton DA, Vinograd J. Complex mitochondrial DNA in leukemic and normal human myeloid cells. Proc Natl Acad Sci USA 1969;62:1077–1084.

Coller HA, Khrapko K, Bodyak ND, Nekhaeva E, Herrero-Jimenez P, Thilly WG. High frequency of homoplasmic mitochondrial DNA mutations in human tumors can be explained without selection. Nat Genet 2001;28:147–150.

Coskun PE, Ruiz-Pesini E, Wallace DC. Control region mtDNA variants: Longevity, climatic adaptation, and a forensic conundrum. Proc Natl Acad Sci USA 2003;100:2174–2176.

Crisan D. Molecular mechanisms in myelodysplastic syndromes and implications for evolution to acute leukemias. Clin Lab Med 2000;20:49–69, viii

Danielson SR, Wong A, Carelli V, Martinuzzi A, Schapira AH, Cortopassi GA. Cells bearing mutations causing Leber's hereditary optic neuropathy are sensitized to Fas-Induced apoptosis. J Biol Chem 2002;277:5810–5815.

de Grey AD. A proposed refinement of the mitochondrial free radical theory of ageing. Bioessays 1997;19:161–166.

Dick JE, Lapidot T. (2005) Biology of normal and acute myeloid leukemia stem cells. Int J Hematol 82(5):389–396. Review.

DiMauro S, Schon EA. Mitochondrial respiratory-chain diseases. N Engl J Med 2003;348:2656–2668.

Donnellan R, Chetty R. Cyclin D1 and human neoplasia (Review). Mol Pathol 1998;51(1):1–7.

Elson JL, Samuels DC, Turnbull DM, Chinnery PF. Random intracellular drift explains the clonal expansion of mitochondrial mutations with age. Am J Hum Genet 2001;68:802–806.

Elson JL, Turnbull JM, Taylor RW. Testing the adaptive selection of mitochondrial haplogroups: an experimental bioenergetics approach. Biochem J 2007;404:e3-e35.
Eng C, Kiuru M, Fernandez MJ, Aaltonea LA. A role for mitochondrial enzymes in inherited neoplasia and beyond. Nat Rev Cancer 2003;3:193–202.
Firkin FC, Clark-Walker GD. Abnormal mitochondrial DNA in acute leukemia and lymphoma. Br J Haematol 1979;43:201–206.
Fliss MS, Usadel H, Cabalero OL, Wu L, Buta M, Eleff SM, Jen J, Sidransky D. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science 2000;287:2017–2019.
Gattermann N. From sideroblastic anemia to the role of mitochondrial DNA mutations in myelodysplastic syndromes. Leuk Res 2000;24:141–151.
Gattermann N, Aul C. Mitochondrial DNA amplification in AML: a hypothesis. Br J Haematol 1997;97:242.
Gattermann N, Aul C, Schneider W. Two types of acquired idiopathic sideroblastic anaemia (AISA). Br J Haematol 1990;74:45–52.
Gattermann N, Berneburg M, Heinisch J, Aul C, Schneider W. Detection of the ageing-associated 5-Kb deletion of mitochondrial DNA in blood and bone marrow of hematologically normal adults. Absence of the deletion in clonal bone marrow disorders. Leukemia 1995;9:1704–1710.
Gattermann N, Retzlaff S, Wang Y-L, Berneburg M, Heinisch J, Wlaschek M, Aul C, Schneider W. A heteroplasmic point mutation of mitochondrial tRNALeu(CUN) in non-lymphoid cell lineages from a patient with acquired idiopathic sideroblastic anaemia. Br J Haematol 1996;93:845–855.
Gattermann N, Retzlaff S, Wang Y-L, Hofhaus G, Heinisch J, Aul C, Schneider W. Heteroplasmic point mutations of mitochondrial DNA affecting subunit I of cytochrome c oxidase in two patients with acquired idiopathic sideroblastic anemia. Blood 1997;90:4961–4972.
Geiger H, van Zant G. The aging of lympho-hematopoietic stem cells. Nat Immunol 2002;3:329–333.
Germing U, Gattermann N, Aivado M, Hildebrandt B, Aul C. Two types of acquired idiopathic sideroblastic anaemia (AISA): a time-tested distinction. Br J Haematol 2000;108:724–728.
Goto Y-I, Horai S, Matsuoka T, Koga Y, Nihei K, Kobayashi M, Nonaka I. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS): a correlative study of the clinical features and mitochondrial DNA mutation. Neurology 1991;42:545–550.
Gutteridge JM. Iron and oxygen: a biologically damaging mixture. Acta Paediatr Scand Suppl 1989;361:78–85.
Habano W, Nakamura S, Sugai T. Microsatellite instability in the mitochondrial DNA of colorectal carcinomas: evidence for mismatch repair systems in mitochondrial genome. Oncogene 1998;17:1931–1937.
Haddad JJ. Redox regulation of pro-inflammatory cytokines and IkappaB-alpha/NF-kappaB nuclear translocation and activation. Biochem Biophys Res Commun 2002;296:847–856.
Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller–Hormelink HK, Vardman J, Lister TA, Bloom Field CD. World Health Organisation classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the clinical advisory committee meeting-Airlie House, Virginia. J Clin Oncol 1999;17:3835–3849.
Hatfill S, Kirby R. Abnormal mitochondrial DNA length polymorphism in patients with the myelodysplastic syndrome. Blood 1994;84:314a.
He L, Luo L, Proctor SJ, Middleton PG, Blakely EL, Taylor RW. Turnbull DM. Somatic mitochondrial DNA mutations in adult-onset leukaemia. Leukemia 2003;17:2487–2491.
Hofhaus G, Gattermann N. Mitochondria harbouring mutant mtDNA - a cuckoo in the nest? Biol Chem 1999;380:871–877.
Hofhaus G, Johns DR, Hurko O, Attardi G, Chomyn A. Respiration and growth defects in transmitochondrial cell lines carrying the 11778 mutation associated with Leber's hereditary optic neuropathy. J Biol Chem 1996;271:13155–13161.
Holme E, Tulinius MH, Larsson N-G, Oldfors A. Inheritance and expression of mitochondrial DNA point mutations. Biochim Biophys Acta 1995;1271:249–252.

Horton TM, Petros JA, Heddi A, Shoffner J, Kaufman AE, Graham SD Jr, Gram Linh T, Wallace DC. Novel mitochondrial DNA deletion found in a renal cell carcinoma. Genes Chromosomes Cancer 1996;15:95–101.

Howell N. Leber hereditary optic neuropathy: respiratory chain dysfunction and degeneration of the optic nerve. Vision Res 1998;38:1495–1504.

Inbal A, Avissar NSM, Kuritzky A, Schejter A, Ben-David E, Shanske S, Garty B-Z. Myopathy, lactic acidosis, and sideroblastic anemia: A new syndrome. Am J Med Genet 1995;55:371–378.

Ivanova R, Lepage V, Loste MN, Schachter F, Wijnen E, Busson M, Cayuela JM, Sigaux F, Charron D. Mitochondrial DNA sequence variation in human leukemic cells. Int J Cancer 1998;76:495–498.

Jeronimo C, Nomoto S, Caballero OL, Usadel H, Henrique R, Varzim G, Oliveira J, Lopes C, Fliss MS, Sidransky D. Mitochondrial mutations in early stage prostate cancer and bodily fluids. Oncogene 2001;20:5195–5198.

Johns DR, Neufeld MJ. Pitfalls in the molecular genetic diagnosis of Leber hereditary optic neuropathy (LHON). Am J Hum Genet 1993;53:916–920.

Jones JB, Song JJ, Hempen PM, Parmigiani G, Hruban RH, Kern SE. Detection of mitochondrial DNA mutations in pancreatic cancer offers a 'mass"-ive advantage over detection of nuclear DNA mutations. Cancer Res 2001;61:1299–1304.

Kraytsberg Y, Nekhaeva E, Bodyak ND, Khrapko K. Mutation and intracellular clonal expansion of mitochondrial genomes: two synergistic components of the aging process? Mech Ageing Dev 2003;124:49–53.

Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med 2000;6:513–519.

Larsson NG, Holme E, Kristiansson B, Oldfors A, Tulinius M. Progressive increase of the mutated mitochondrial DNA fraction in Kearns-Sayre syndrome. Pediatr Res 1991;28:131–133.

Liang Y, Van Zant G, Szilvassy SJ. Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. Blood 2005;106:1479–1487.

Linnartz B, Anglmayer R, Zanssen S. Comprehensive scanning of somatic mitochondrial DNA alterations in acute leukemia developing from myelodysplastic syndromes. Cancer Res 2004;64:1966–1971.

Liu VW, Shi HH, Cheung AN, Chiu PM, Leung TW, Nagley P, Wong LC, Ngan HY. High incidence of somatic mitochondrial DNA mutations in human ovarian carcinomas. Cancer Res 2001;61:5998–6001.

Liu SL, Lin X, Shi DY, Cheng J, Wu CQ, Zhang YD. Reactive oxygen species stimulate human hepatoma cell proliferation via cross-talk between PI3K/PKB and JNK signaling pathways. Arch Biochem Biophys 2002;406:173–182.

Luft R. The development of mitochondrial medicine. Proc Natl Acad Sci USA 1994;91:8731–8738.

Marley SB, Lewis JL, Davidson RJ, Roberts IAG, Dokal I, Goldman JM, Gordon MY. Evidence for a continuous decline in haematopoietic cell function from birth: application to evaluating bone marrow failure in children. Br J Haematol 1999;106:162–166.

McShane MA, Hammans SR, Sweeney M, Holt IJ, Beattie TJ, Brett EM, Harding AE. Pearson syndrome and mitochondrial encephalomyopathy in a patient with a deletion of mtDNA. Am J Hum Genet 1991;48:39–42.

Miguel A, Hernández-Yago J, Knecht E, Renau-Piqueras J. A comparative study of complex mitochondrial DNA in human lymphocytes transformed by Epstein-Barr virus and PHA. Acta Haematologica 1982;68:96–104.

Modica-Napolitano JS, Kulawiec M, Singh KK. Mitochondria and human cancer (Review). Curr Mol Med 2007;7(1):121–31.

Morrison SJ, Wandycz AM, Akashi K, Globerson A, Weissman IL. The aging of hematopoietic stem cells. Nat Med 1996;2:1011–1016.

Muller-Hocker J. Random cytochrome-C-oxidase deficiency of oxyphil cell nodules in the parathyroid gland. A mitochondrial cytopathy related to cell ageing? Pathol Res Pract 1992;188:701–706.

Murdock DG, Christiacos NC, Wallace DC. The age-related accumulation of a mitochondrial DNA control region mutation in muscle, but not brain, detected by a sensitive PNA-directed PCR clamping based method. Nucleic Acids Res 2000;28:4350–4355.

Nekhaeva E, Bodyak ND, Kraytsberg Y, McGrath SB, Van Orsouw NJ, Pluzhnikov A, Wei JY, Vijg J, Khrapko K. Clonally expanded mtDNA point mutations are abundant in individual cells of human tissues. Proc Natl Acad Sci USA 2002;99:5521–5526.

Nelson I, Bonne G, Degoul F, Marsac C, Ponsot G, Lestienne P. Kearns-Sayre syndrome with sideroblastic anemia: molecular investigations. Neuropediatrics 1992;23:199–205.

Nikoselainen E, Asola M, Kalimo H, Savontaus ML, Majander A. Benzodiazepine sensititvity in Leber's hereditary optic neuropathy (letter). Lancet 1992;340:1223–1224.

Okochi O, Hibi K, Uemura T, Inoue S, Takeda S, Kaneko T, Nakao A. Detection of mitochondrial DNA alterations in the serum of hepatocellular carcinoma patients. Clin Cancer Res 2002;8:2875–2878.

Parker JE, Fishlock KL, Mijovic J, Czepulkowski B, Pagliuca A, Mufti GJ. 'Low-risk' myelodysplastic syndrome is associated with excessive apoptosis and an increased ratio of pro- versus anti-apoptotic bcl-2-related proteins. Br J Haematol 1998;103:1075–1082.

Parrella P, Xiao Y, Fliss M, Sanchez-Cespedes M, Mazzarelli P, Rinaldi M, Nicol T, Gabrielson E, Cuomo C, Cohen D, Pandit S, Spencer M, Rabitti C, Fazio VM, Sidransky D. Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. Cancer Res 2001;61:7623–7626.

Pearson HA, Lobel JS, Kocoshis SA, Naiman JL, Windmiller J, Lammi AT, Hoffman R, Marsh JC. A new syndrome of refractory sideroblastic anemia with vacuolization of marrow precursors and exocrine pancreatic dysfunction. J Pediatr 1979;95:976–984.

Penta JS, Johnson FM, Wachsman JT, Copeland WC. Mitochondrial DNA in human malignancy. Mutat Res 2001;488:119–133.

Petros JA, Baumann AK, Ruiz-Pesini E, Amin MB, Sun CQ, Hall J, Lim S, Issa MM, Flanders WD, Hosseini SH, Marshall FF, Wallace DC. mtDNA mutations increase tumorigenicity in prostate cancer. Proc Natl Acad Sci USA 102(3):719–724.

Polyak K, Li Y, Zhu H, Lengauer C, Willson JKV, Markowitz SD, Trush MA, Kinzler KW, Vogelstein B. Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet 1998;20:291–293.

Poulton J, Morten K. Noninvasive diagnosis of the MELAS syndrome from blood DNA. Ann Neurol 1993;34:116.

Puigserver P, Spiegelmann BM. Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. 2003;24(1):78–90.

Rahman S, Poulton J, Marchington D, Suomalainen A. Decrease of 3243 A→G mtDNA mutation from blood in MELAS syndrome: a longitudinal study. Am J Hum Genet 2001;68:238–240.

Rawles JM, Weller RO. Familial association of metabolic myopathy, lactic acidosis, and sideroblastic anemia. Am J Med 1974;56:891–897.

Rötig A, Cormier V, Blanche S, Bonnefont J-P, Ledeist F, Romero N, Schmitz J, Rustin P, Fischer A, Saudubray J-M, Munnich A. Pearson's marrow-pancreas syndrome. A multisystem mitochondrial disorder in infancy. J Clin Invest 1990;86:1601–1608.

Rötig A, Cormier V, Koll F, Mize CE, Saudubray J-M, Veerman A, Pearson HA, Munnich A. Site-specific deletions of the mitochondrial genome in the Pearson marrow-pancreas syndrome. Genomics 1991;10:502–504.

Rustin P, Rotig A. Inborn errors of complex II-unusual human mitochondrial diseases. Biochim Biophys Acta 2002;1553:117–122.

Sakamaki T, Casimiro MC, Ju X, Quong AA, Katiyar S, Liu M, Jiao X, Li A, Zhang X, Lu Y, Wang C, Byers S, Nicholson R, Link T, Shemluck M, Yang J, Fricke ST, Novikoff PM,

Papanikolaou A, Arnold A, Albanese C, Pestell R. Cyclin D1 determines mitochondrial function in vivo. Mol Cell Biol 2006;26(14):54495469.
Santoro A, Salvioli S, Raule N, Capri M, Sevini F, Valensin S, Monti D, Bellizzi D, Passarino G, Rose G, De Benedictis G, Franceschi C. Mitochondrial DNA involvement in human longevity. Biochim Biophys Acta 2006;757(9–10):1388–1399.
Schumacher HR, Szekely IE, Patel SB, Fisher DR. Leukemic mitochondria. I. Acute myeloblastic leukemia. Am J Pathol 1974;74:71–82.
Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, wans Field KD, Pan Y, Simon MC, Thompson CB, Gottleb E. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell 2005;7:77–85.
Shin MG, Kajigaya S, Levin BC, Young NS. Mitochondrial DNA mutations in patients with myelodysplastic syndromes. Blood 2003;101:3118–3125.
Shin MG, Kajigaya S, Tarnowka M, McCoy Jr JP, Levin BC, Young NS. Mitochondrial DNA sequence heterogeneity in circulating normal human CD34 cells and granulocytes. Blood 2004;103(12):4466–4471.
Shoubridge EA, Karpati G, Hastings KEM. Deletion mutants are functionally dominant over wild-type mitochondrial genomes in skeletal muscle fiber segments in mitochondrial disease. Cell 1990;62:43–49.
Singh G, Lott MT, Wallace DC. A mitochondrial mutation as a cause of Leber's hereditary optic neuropathy. N Engl J Med 1989;36:1300–1305.
Singh KK, Kulawiec M, Still I, Desouki MM, Geradts J, Matsui S. Inter-genomic cross talk between mitochondria and the nucleus plays an important role intumorigenesis.Gene 2005;354:140–146.
Singh KK Mitochondria damage checkpoint, aging, and cancer (Review). Ann NY Acad Sci 2006;1067:182–190.
Taylor RW, Barron MJ, Borthwick GM, Gospel A, Chinnery PF, Samuels DC, Taylor GA, Plusa SM, Needham SM, Greaves LC, Kirkwood TBL, Turnbull DM. Mitochondrial DNA mutations in human colonic crypt stem cells. J Clin Invest 2003;112:1351–1360.
Tehranchi R, Fadeel B, Forsblom A-M, Christensson B, Zhivotovsky B, Hellström-Lindberg E. Evidence for a role of mitochondrial signaling in apoptosis in low-risk myelodysplastic syndromes. Blood 2001;98:730a.
Verma M, Naviaux RK, Tanaka M, Kumar D, Franceschi C, Singh KK. Meeting report: mitochondrial DNA and cancer epidemiology. Cancer Res 2007;67(2):437–439.
Wallace DC. Mitochondrial DNA mutations and neuromuscular disease. Trends Genet 1989;5:9–13.
Wallace DC. Mitochondrial genetics: a paradigm for aging and degenerative diseases? Science 1992;256:628–632.
Wallace DC. Mitochondrial diseases in man and mouse. Science 1999;283:1482–1488.
Wallace DC. A mitochondrial paradigm of Metabolic and degenerative disease, aging, and cancer. Annu Rev Genet 2005;39:359–407.
Wang Y-L, Choi H-K, Heinisch J, Aul C, Gattermann N. The MERRF mutation of mitochondrial DNA in the bone marrow of a patient with acquired idiopathic sideroblastic anemia. Am J Hematol 1999;60:83–84.
Warburg O. On the origin of cancer cells. Science. 1956;123:309–314.
Wickramasinghe SN, Chalmers DG, Cooper EH. A study of ineffective erythropoiesis in sideroblastic anaemia and erythraemic myelosis. Cell Tissue Kinet 1968;1:43–50.
Wickramasinghe SN, Hughes M. Capacity of ringed sideroblasts to synthesize nucleic acids and protein in patients with primary acquired sideroblastic anaemia. Br J Haematol 1978;38:345–352.
Wolvetang EJ, Johnson KL, Krauer K, Ralph S, Linnane AW. Mitochondrial respiratory chain inhibitors induce apoptosis. FEBS Lett 1994;339:40–44.

Yao YG, Ellison FM, McCoy JP, Chen J, Young NS. Age-dependant accumulation of mtDNA mutations in murine hematopoietic stem cells is modulated by the nuclear genetic background. Hum Mol Gen 2007;16:286–294.

Zanssen S, and Buse G. Successful chemotherapy in a male patient with malignant lymphoma and Leber's hereditary optic neuropathy (LHON). Am J Hematol 2003;72:263–266.

Zanssen S, Gunawan B, Fuzesi L, et al. Renal oncocytomas with rearrangements involving 11q13 contain breakpoints near CCND1. Cancer Genet Cytogenet 2004;149:120–124.

Zanssen, and Schon (2005) Mitochondrial DNA mutations in cancer. PLOS Med 11:e401.

Mitochondria and Oncocytomas

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Oncocytic Tumors and Oncocytomas

The designation oncocytoma was coined by Hamperl (1962) to describe a tumor composed by "oncocytes." Oncocyte is a descriptive term for a neoplastic or non-neoplastic cell stuffed with mitochondria that give a grainy, eosinophilic appearance to its swollen cytoplasm. In many instances, oxyphilic transformation is used as a synonym for oncocytic transformation, thus leading to the utilization of oxyphilic tumor as a synonym for oncocytic tumor or oncocytoma. In the thyroid, other terms are used: Hürthle cell transformation and Hürthle cell tumors (Nesland et al. 1985; Sobrinho-Simoes et al. 1985, 2005). Finally, there are, in some organs, tumors composed by oncocytes that carry specific designations (e.g. Warthin's tumor of the salivary glands).

In this chapter we will only focus on neoplastic oncocytes, i.e., on the tumors – oncocytomas – composed by such cells, but they may also occur in several "normal" organs of elderly patients (e.g. parathyroid glands), as well as in inflammatory autoimmune disorders (e.g. Hashimoto's thyroiditis) and in hyperplastic conditions (e.g. adenomatous goiter displaying oncocytic transformation).

To be an oncocytic tumor is not a black and white phenomenon. It takes many years before the accumulation of mitochondria reaches the "oncocytic" threshold. In the thyroid, like in the kidney, mitochondrion-rich tumors have been described (Sobrinho-Simoes et al. 1985). The designation oncocytoma is usually used for characterizing a benign tumor, although some oncocytomas may occasionally follow a malignant course, for instance giving rise to metastases. The malignant counterpart of an oncocytoma is sometimes designated as a malignant oncocytoma. Whenever one is dealing with epithelial neoplasms, such malignant tumors may also be designated oncocytic, or oxyphilic, or Hürthle cell carcinomas. The great majority of oncocytomas – from now on we will use oncocytoma as the umbrella descriptive term for all tumors composed of mitochondrion-rich cells and oncocytic cell to designate the mitochondrion-rich cell – are epithelial-derived tumors, but

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Table 1 Papers, in English, reporting the occurrence and the characteristics of oncocytic tumors and/or oncocytomas outside the thyroid gland published in the last 55 years[a]

Oncocytomas				
Organ	Total number of papers	Papers with data on mtDNA/ mitochondrial proteins	Organ	Total number of papers
Kidney	277	12	Choroid plexus	1
Salivary gland	68	3	Colon	1
Parathyroid	39	3	Epiglottis	1
Globe, eyelid and conjunctiva	28		Ethmoid sinus	1
Adrenal gland	19		Oesophagus	1
Nasal cavity, nasopharynx, and larynx	17		Ovary	1
Hypophysis	12		Pancreas	1
Lung	10	2	Prostate	1
Maxillary sinus	4		Skin	1
Bronchus	4		Soft tissue	1
Breast	3	1	Stomach	1
Spinal cord	3		Tongue	1
Palate	2		Trachea	1

Total number of papers: 499; total number of papers with data on mtDNA/mitochondrial proteins: 21
[a]Besides the terms oncocytomas and oncocytic tumors we have also searched in the web the terms oxyphilic and Hürthle cell tumors and, in salivary glands, the designation Warthin's tumor

there are also on record examples of oncocytomas occurring in nonepithelial settings. In Table 1 we have summarized the different sites, other than the thyroid, where oncocytomas have been reported to occur in 499 papers published in English in the last 55 years. During the same period, more than 600 papers on thyroid oncocytomas (Hürthle cell tumors, usually) have been published.

Oncocytomas represent a unique model to study mitochondrial alterations and their role in tumorigenesis because of the huge amount of abnormal mitochondria accumulated in the cytoplasm of their cells. Even though these tumors may occur in any organ, the endocrine system and some parenchymatous organs appear to be particularly susceptible to their development. The thyroid is, by far, the most frequent site for the occurrence of oncocytomas (more than 600 papers on record in the English literature). As pointed in Table 1, oncocytomas have most frequently been reported, after the thyroid, in kidney, salivary glands and parathyroid and rarely found, for instance, in the gastrointestinal tract. The abundance of mitochondria is also phenotypically reflected from the color standpoint, and oncocytomas usually have a brown color owing to their high cytochrome content.

Understanding the mechanisms behind the large accumulation of mitochondria in the cytoplasm of these tumor cells remains to be clarified. The putative relationship

between the accumulation of mitochondria and tumourigenesis remains also a major challenge.

Mitochondrial DNA (MtDNA) and Human (degenerative and neoplastic) Disorders

The primary and well known function of mitochondria is to provide the cell with energy in the form of ATP, which is achieved through the oxidative phosphorylation (OXPHOS) system. Any dysfunction of this system presumably results in deficient ATP production, which could result in cell death. Therefore, any deleterious mutation in the 13 peptide-encoding genes of mtDNA is most likely to impair ATP production, since they all encode OXPHOS peptides. In fact, several mtDNA mutations have been associated to degenerative diseases, sometimes also called mitochondrial diseases, which typically affect high-energy requiring tissues, such as the brain and muscle (Taylor and Turnbull 2005). Curiously, in some of these degenerative diseases the microscopic study has revealed the oncocytic transformation of the involved cells, with an increased number of mitochondria, as well as mitochondrial structural abnormalities (Lindal et al. 1992; Sengers, Stadhouders and Trijbels 1984). Previous studies have described the oncocytic (oxyphilic) transformation of epithelial cells of the choroid plexus in mitochondrial encephalopathies (due to mtDNA alterations) (Kepes 1983), as well as the cells involved in Leigh syndrome (Kepes 1983), MELAS (Ohama and Ikuta 1987) and Kearns-Sayre syndrome (Tanji et al. 2000), which are all degenerative disorders arising as a consequence of mitochondrial OXPHOS dysfunction.

In addition to degenerative diseases, where mtDNA mutations have been proven to be causative, mtDNA alterations – including point mutations, insertions, deletions and complex rearrangements of the mtDNA molecule – have also been found in a variety of human tumors with and without oncocytic features (for a review see ref. Czarnecka, Golik and Bartnik 2006). The point mutations, insertions and deletions seem to occur throughout the mtDNA, with no particular hotspot, with the exception of the displacement-loop (D-loop), particularly the homopolymeric "C" tract between positions 303 and 309 [Cambridge reference sequence (MITOMAP)], where insertions and/or deletions are particularly frequent (Maximo et al. 2005b; Sanchez-Cespedes et al. 2001). Most of the studies on mtDNA alterations occurring in human tumors have been essentially descriptive, whereas the putative functional role of such alterations has only recently been addressed.

Mitochondria and Oncocytic Tumors of the Thyroid (Hürthle cell tumors)

Oncocytic thyroid tumors (Fig. 1) are, probably, those that are better studied in terms of mtDNA. A specific mtDNA alteration – a large deletion encompassing 4977 bp of mtDNA – known as the mtDNA common deletion (CD), is often found

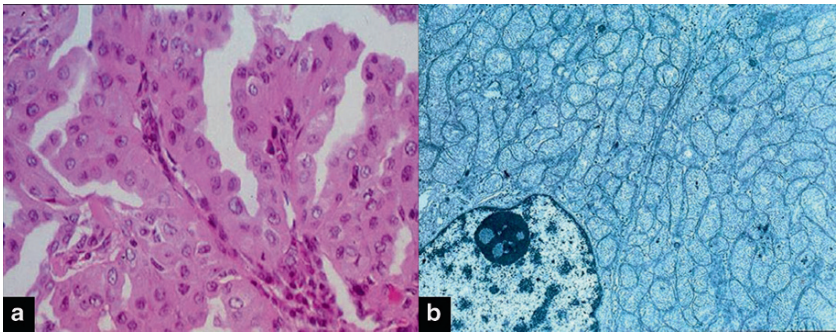

Fig. 1 Thyroid oncocytomas (Hürthle cell tumors). (**a**) The neoplastic cells have an abundant, pink and granular cytoplasm. The papillary growth pattern and the nuclear characteristics are typical of an *oncocytic variant of a papillary thyroid carcinoma* (Hematoxilin and eosin – H&E, ×400). (**b**) This electron microscopy of an *oncocytic variant of a follicular thyroid tumor* shows a neoplastic cell filled with mitochondria, some of which contain dense bodies. Notice the prominent nucleolus (uranyl and acetate and lead citrate – ua/lc, ×9200) (See Color Insert)

in oncocytic thyroid tumors and was proposed as a hallmark of oncocytic thyroid tumors (Maximo et al. 1998; Maximo and Sobrinho-Simoes 2000b; Maximo and Sobrinho-Simoes 2000a). This deletion removes seven OXPHOS genes (*ATPase6*, *ATPase8*, *COIII*, *ND3*, *ND4L*, *ND4* and *ND5*) and five tRNAs (glycine, arginine, histidine, serine and leucine), thus resulting in severe impairment of the OXPHOS system.

In a large study conducted by Máximo et al. (2002), the mtDNA CD was found in every thyroid tumor with oncocytic features, irrespective of the histological subtype [papillary (PTC) and follicular thyroid carcinomas (FTC) or follicular thyroid adenomas (FA)]. The mtDNA CD was also present in non-oncocytic thyroid tumors, but with a significantly lower frequency (18.8% of PTC, 0% of FTC and 33.3% of FA) (Maximo et al. 2002). The relative amount of deleted mtDNA molecules within each tumor was also assessed and found to be significantly higher ($p = 0.0001$) in oncocytic thyroid tumors (range: 2.7–9.6%) than in non-oncocytic thyroid tumors (range: 0–1.2%) (Maximo et al. 2002).

Traditionally, the association between mtDNA CD and oncocytic phenotype has been explained through a positive feedback mechanism: the severe impairment of the OXPHOS system (as a consequence of the mtDNA CD) would engage and activate nuclear genes that control mitochondrial number, resulting in an increase in the mitochondrial mass (Attardi et al. 1995; Heddi et al. 1996).

The prevalence of mtDNA mutations was also assessed by Máximo et al. (2002) in a large series of oncocytic (20 FA, 13 FTC and 10 PTC) and non-oncocytic (15 FA, 5 FTC and 16 PTC) thyroid tumors, through direct sequencing of about 70% of the mitochondrial genome. The aforementioned "C" tract in the D-loop region showed a high frequency of somatic mutations – 32 of 66 tumors (48.5%) – which were present in similar levels in all types of tumors (Maximo et al. 2002). In addition to D-loop, 57 somatic mutations were also detected in 34 of the 66 thyroid

tumors (51.5%). These mutations included frameshift (three) and point mutations (51, of which 25 were silent and 26 were missense mutations) present in all genes that encode OXPHOS proteins (except *ATPase8*), as well as three mutations in three tRNAs (serine, alanine and isoleucine) (Maximo et al. 2002). Missense somatic mutations in complex I genes (without any apparent concentration in a single gene) were more frequently detected in malignant tumors than in adenomas ($p = 0.015$) and a significant association was also observed between D-loop somatic mutations and the occurrence of somatic mutations in other mtDNA genes ($p = 0.026$) (Maximo et al. 2002). In Fig. 2 we have summarized all but silent mtDNA somatic mutations reported to date in oncocytic tumors. A large number ($n = 253$) of mtDNA variants (alterations present both in tumor and adjacent thyroid tissue) were disclosed in all tumor types. The variants affecting genes of complex I and IV were significantly more frequent in patients with malignant tumors than in patients with benign tumors, whereas those affecting complex V genes – almost all in *ATPase6* (34/37), most of them missense (27/34) – were associated with the presence of oncocytic features in the tumors of the patients (Maximo et al. 2002).

The mtDNA status was further analyzed by Gasparre et al. (2007) in a series of 50 oncocytic lesions, of which 45 occurred in thyroid (16 classified as oncocytic hyperplastic thyroid nodules, seven as oncocytic follicular adenomas and 22 as oncocytic thyroid carcinomas) and five were oncocytic breast tumors. The authors found that 26 of the 45 (57.8%) oncocytic thyroid samples harboured 30 somatic mtDNA mutations, of which 25 were located in complex I genes (Gasparre et al. 2007) (Fig. 2). In 12 of 45 (26.7%) cases the mutations were considered as disruptive, i.e., they were either frameshift or nonsense, and they were all located in complex I genes (Gasparre et al. 2007) (Fig. 2). In addition, 21 non-oncocytic cases were also analyzed, as controls. It was observed that eight of them (38.1%) harbored mtDNA somatic mutations and only two (9.5%) presented disruptive mutations, both occurring in complex I genes (Gasparre et al. 2007). In accordance with the results obtained in oncocytic thyroid tumors, the only breast tumor that presented a disruptive somatic mtDNA mutation (also located in a complex I gene) was a mitochondrion-rich tumor (Fig. 2). To address the correlation between mtDNA mutations and oncocytic phenotype, the authors established primary cultures from two thyroid tumors, each with a disruptive mtDNA mutation. Intriguingly, none of the primary cultures showed evidence of the disruptive mtDNA mutations found in the original biopsies and, moreover, the oncocytic phenotype was lost during culture (Gasparre et al. 2007). The authors suggested that, under the culture conditions used, the aforementioned mtDNA mutations are negatively selected (Gasparre et al. 2007).

The results of Gasparre et al. (2007) associate the presence of disruptive mtDNA complex I mutations with the oncocytic phenotype of thyroid tumors. In line with the rationale applied for the association of the mtDNA CD with oncocytic features – severe impairment of the OXPHOS system due to the mtDNA CD would lead to a positive feedback in the nucleus, resulting in accumulating dysfunctional mitochondria – the disruptive complex I mutations found in a subset of oncocytic thyroid

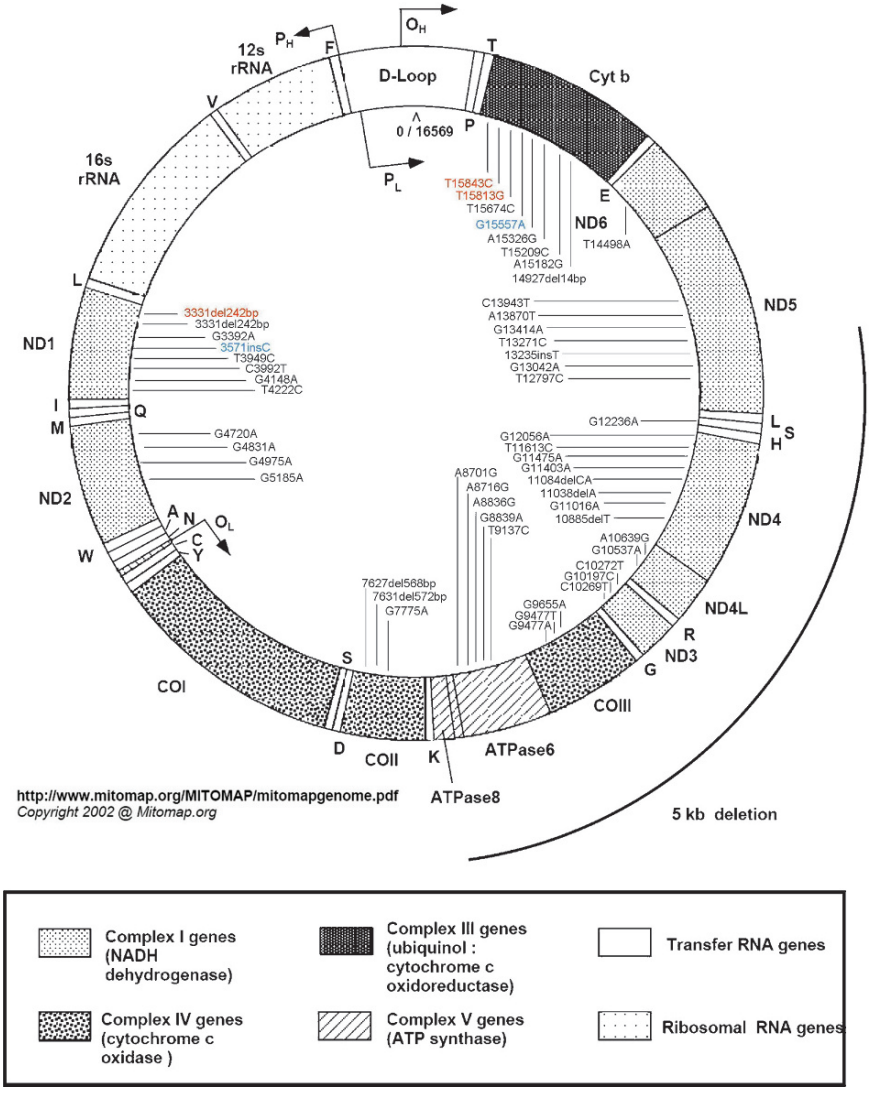

Fig. 2 Schematic representation of the mtDNA somatic mutations found in human oncocytomas and in the XTC.UC1 thyroid oncocytoma cell line. Only missense, nonsense or frameshift mutations are depicted. Numbers indicate the mutation position according to the Cambridge reference sequence; mutations in thyroid oncocytomas are represented in black, in breast oncocytomas in red and in the thyroid oncocytoma cell line in blue. The 5kb deletion was found in thyroid oncocytomas, as well as in Warthin's tumor. Picture taken and adapted with permission from MITOMAP (See Color Insert)

tumors may also result in OXPHOS impairment and, subsequently, to accumulation of abnormal mitochondria.

In addition to genetic studies, biochemical analyses of oncocytic thyroid tumors have revealed that the ATP synthesis in oncocytic tumor cells is impaired. Savagner

et al. (2001), studied seven fresh oncocytic thyroid tumors and respective controls, having found that the ATP synthesis was lower in all seven tumors, with a parallel overexpression of uncoupling protein 2 (UCP2), which is a protein that uncouples the electron flow in the OXPHOS system from the ATP production in complex V. The authors also found that two mitochondrial genes – *ND2* and *ND5* – were overexpressed in relation to normal thyroid tissue, being the mtRNA/mtDNA ratio 4:1 when compared to normal thyroid tissue (Savagner et al. 2001). Confirming these observations, two other studies, using microarrays, have found that the majority of the peptide-encoding mtDNA genes were overexpressed in thyroid oncocytic tumors (Baris et al. 2004; Jacques et al. 2005). These results suggest that the defective ATP production observed may explain the mitochondrial proliferation in the tumor cells.

Using differential display it was disclosed, among other alterations, an overexpression of the gene encoding the Core I subunit of the complex III of the mitochondrial OXPHOS system in a follicular carcinoma composed of Hürthle cells (Maximo et al. 2004). However, in a large series of thyroid tumors, *Core I* overexpression was found to be associated with benign and malignant tumors of the thyroid with microfollicular growth pattern, independently of the presence of Hürthle cells (Maximo et al. 2004).

The establishment of a thyroid oncocytic cell line – XTC.UC1 – has allowed to study, in vitro, the oncocytic phenotype. The XTC.UC1 harbors a frameshift mutation in the *ND1* gene (complex I) and a missense mutation in the *CytB* gene (complex III). Bonora et al. (2006b) analyzed this cell line and found that it displays a marked reduction in the enzymatic activity of complexes I and III, an observation that is in accordance with the XTC.UC1 mtDNA mutations, which may also explain the enhanced production of ROS observed in XTC.UC1 cell line (Bonora et al. 2006b). To discriminate whether the impairment of complexes I and III depended on mitochondrial or nuclear DNA mutations, the authors generated osteosarcoma-derived cybrids carrying the XTC.UC1 mtDNA; these cybrids showed a similar behaviour to that of XTC.UC1 cell line, pointing to the functional role of the mtDNA mutations (Bonora et al. 2006b).

The XTC.UC1 cell line may be an extremely important tool to study both the oncocytic phenotype and also the functional role of mtDNA mutations. In fact, the presence of a frameshift mutation in a complex I gene – *ND1* – confirms the in vivo observations of oncocytic thyroid tumors, that often display disruptive complex I mutations (Gasparre et al. 2007). Using the cybrid technology, it has been shown that the *ND1* and *CytB* mutations in XTC.UC1 cell line have a functional consequence in the OXPHOS system, since they result in marked reduction of complex I and complex III, respectively (Bonora et al. 2006b). It remains to be elucidated if these alterations play an active role in tumor development and, if so, what are the mechanisms involved.

Chromosomal abnormalities are frequently detected in oncocytic thyroid tumors (Tallini et al. 1999; Wada et al. 2002). Detailed studies have revealed three loci – 9q13-q21.3, 17p13 and 19p13.2 – that appear to be specifically associated and may comprise genes related to the development of oncocytic thyroid tumors (Canzian

et al. 1998; Farrand et al. 2002; Frisk et al. 1999). It remains to be found the putative relationship between most of the chromosomal alterations and the mitochondrial alterations that are the hallmark of oncocytomas. Besides the association between *GRIM-19* somatic mutations and oncocytic thyroid tumors (see below), it remains to be found if mutations in other nuclear genes encoding mitochondrial proteins may be associated with the occurrence of oncocytomas.

It has been shown that the oncocytic features, per se, do not allow a diagnosis of malignancy or benignity, although it is well known that such accumulation of mitochondria indicates a low proliferative turn-over and is thus associated, in most instances, to benign neoplasms or malignant tumors of low malignancy. With regard to oncocytic cell tumors of the thyroid, they encompass benign lesions – the so-called oncocytic or oncocytic cell variant of adenomatous goiter and adenoma – and malignant lesions. The latter are divided according to the cell of origin and the histotype into oncocytic, or oncocytic cell variant, of papillary, follicular, poorly differentiated and medullary carcinoma (DeLellis et al. 2004).

Mitochondria and Renal Oncocytomas

Renal oncocytomas are usually benign tumors, that are thought to originate from the collecting duct, and account for 3–7% of all renal tumors (Zambrano et al. 1999). Like the oncocytic thyroid tumors, renal oncocytomas are morphologically characterized by the accumulation of abnormal shaped mitochondria in the cytoplasm (Fig. 3)

Few publications have analyzed mtDNA alterations in renal oncocytomas. Welter et al. (1989) used restriction endonucleases to search for mtDNA abnormalities in six

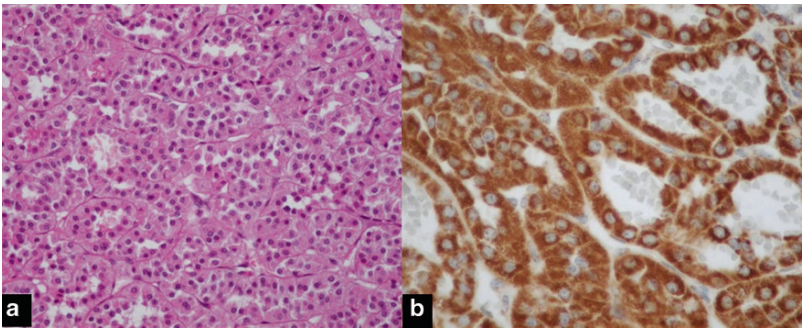

Fig. 3 Renal oncocytoma. (**a**) Overview of a tumor with tubular growth pattern, in which all the neoplastic cells display oncocytic features (H&E, ×200). (**b**) The immunocytochemical study, using an antibody antisuccinate dehydrogenase A (SDHA), which is a subunit of mitochondrial complex II of the OXPHOS system, discloses the presence of a large number of mitochondria in the neoplastic cells (anti-SDHA, ×400) (See Color Insert)

renal oncocytomas and observed that every tumor displayed an extra band, which was not noted in the corresponding normal tissue; however, these findings were not reproduced by Brooks et al. (1999), who did not observe any mtDNA alterations in five renal oncocytomas. Tallini et al. (1994), searched for alterations in *COXI* and D-loop region, but did not find any alterations in 10 renal oncocytomas. In addition, these authors also analyzed the presence of the mtDNA CD, but failed to detect an increased frequency in comparison to controls (Tallini et al. 1994). A significant difference was observed in the content of mtDNA, which was five-fold higher in oncocytomas than in normal controls, but this fact could easily be expected given the accumulation of mitochondria in the cytoplasm (Tallini et al. 1994).

Simmonet et al. (2002), have analyzed the status of the OXPHOS system in a series of 25 renal cell carcinomas (RCC), including 16 clear cell RCC, five papillary RCC and four oncocytomas. In each case, the authors analyzed the activities of complexes II–V in tumor and normal tissue. Clear-cell and papillary RCC showed a general decrease in the activity of all analyzed complexes, when compared to the respective normal tissue; moreover, OXPHOS impairment increased from the less aggressive to the most aggressive RCCs (Simonnet et al. 2002). At variance with these findings, renal oncocytomas displayed a normal or slightly elevated activity of complexes II–V, whereas complex I was not detectable in two-dimension electrophoresis; in addition, renal oncocytomas also showed a five fold increase in citrate synthase (an indicator of mitochondrial proliferation) (Simonnet et al. 2002). The absence of complex I, together with an increase in the remaining complexes and citrate synthase led the authors to conclude that the mitochondrial proliferation in renal oncocytomas might be a compensatory mechanism for a decreased OXPHOS activity (Simonnet et al. 2002).

To test whether the accumulated mitochondria in renal oncocytomas are functional or not, Simonnet et al. (2003) examined the status of OXPHOS complex I, which was previously proposed to be deficient in these tumors. The authors found that complex I was selectively deficient, both at the activity and protein level, whereas the other OXPHOS complexes were either normal or upregulated (Simonnet et al. 2003). This complex I decrease was also observed in other types of renal tumors, but, in contrast to renal oncocytomas, the remaining types of renal tumors also showed a decrease in the other OXPHOS complexes (Simonnet et al. 2003).

We studied the mtDNA of 14 renal oncocytomas looking for the mtDNA CD, as well as for alterations in D-loop and Complex V genes (*ATPase6* and *ATPase8*) (Portugal et al., unpublished observations). The mtDNA CD was present in 11 (78.6%) of 14 renal oncocytomas and in seven (50%) of the 14 cases in the respective adjacent "normal" parenchyma. This alteration was also detected in two cases of RCC synchronous with renal oncocytomas.

Mutations in the D-loop region were seen in six (42.9%) of the 14 oncocytomas; the alterations were located in the repetitive regions D310, D514 and D568. Four somatic mutations in the OXPHOS Complex V genes were detected in three renal oncocytomas (21.4%); of those mutations, three were in *ATPase6* – two missense mutations (T8696C and G9055A – 14.3%) and one silent mutation (G8697A).

The chromosomal alterations identified in renal oncocytomas are heterogeneous and usually characterized by structural rearrangements comprising the breakpoint region 11q13, combined losses of chromosomes 1 and Y, losses of chromosomes 14, 1 or 1p, other monosomies, trisomies and loss of heterozigosity in several chromosomes (Zambrano et al. 1999). Alchanati et al. (2006) reported that expression of *GRIM-19* is lost or severely depressed in a number of primary RCC regardless of the type of carcinoma, but they did not study renal oncocytomas. Like oncocytic cell tumors of the thyroid (see below), renal oncocytomas may also occur in familial forms, in the context of Birt Hogg Dubé (BHD) syndrome (Pavlovich et al. 2002).

Mitochondria and Oncocytic Tumors of the Salivary Glands (Warthin's tumor)

Warthin's tumor is the second most common salivary gland neoplasm, arising almost always in the parotid gland, usually in the fifth to seventh decades of life. Some are multifocal and about 10% are bilateral. Smokers have approximately eight times more risk for developing these tumors than nonsmokers. Warthin's tumors are constituted by cystic spaces, limed by a double layer of neoplastic epithelial cells, having oncocytic features and resting on a lymphoid stroma (Fig. 4).

There are few studies assessing mtDNA alterations in the normal parotid epithelial cells and in the oncocytic cells of Warthin's tumors. Lewis et al. (2000) showed, using two-color FISH, that all oncocytic cells in Warthin's tumors contained

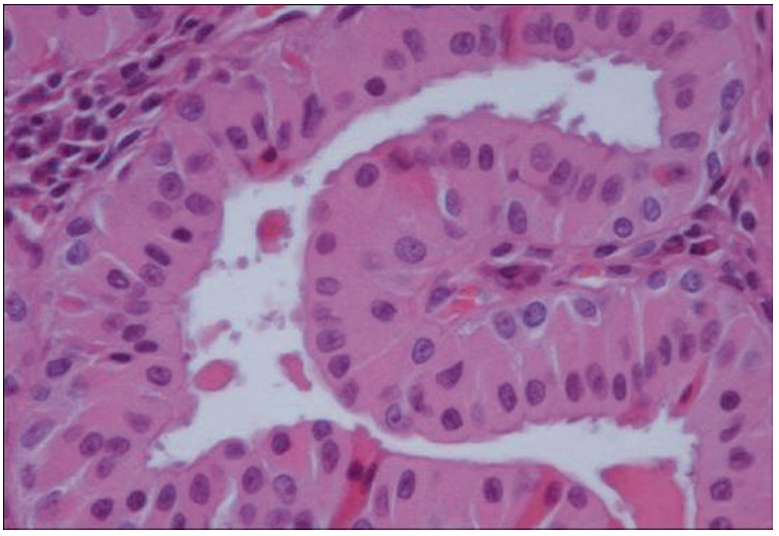

Fig. 4 Salivary gland oncocytoma (Warthin's tumor). High magnification showing neoplastic cells with prominent oncocytic features. Notice the foci of lymphocytic infiltration (H&E, ×600) (See Color Insert)

mitochondria showing a reduction on mtDNA signal, in comparison to the control signal, and that all oncocytic cells had mixed populations of normal and deleted mtDNA (heteroplasmy), but no cells had exclusively deleted mtDNA. Lewis et al. (2000) also found the presence of a low level of mtDNA deletions in normal parotid epithelial cells, a finding that supports the assumption that these deletions may precede the oncocytic phenotype.

The majority of patients with Warthin's tumors have a history of cigarette smoking. Lewis et al. (2002) studied the level of the mtDNA CD in normal parotid tissue from smokers and nonsmokers, having found that the mtDNA CD is present in the parotid gland, even in young individuals, and that its level increases with age. The authors did not find an increase in the level of the mtDNA CD in the parotid gland of smokers relatively to nonsmokers (Lewis et al. 2002). Nonetheless, there was a specific base substitution in the *ND2* gene in the parotid gland of smokers, supporting an increased level of oxidative damage in smokers, and providing a model for demonstrating a putative smoking associated pattern of mutagenesis (Lewis et al. 2002).

The relation between cigarette smoking and mtDNA alterations has been confirmed in a study on lung lesions. The study involved 37 patients with lung cancer and revealed that the mtDNA CD was present in 20 of the 37 tumors (54.1%) and in 22 of 37 (59.5%) adjacent normal lung tissue (Dai et al. 2006). The authors did not refer whether or not the tumors with mtDNA CD had oncocytic features. In addition, the analysis of 20 histologically normal lung tissue samples in subjects without lung cancer revealed that six of them (30.0%) also harbored the mtDNA CD (Dai et al. 2006). The high frequency of mtDNA CD in lung tissue adjacent to the tumors suggests that this lesion was already present prior to the development of the tumors. Moreover, the finding of a positive association between cigarette smoking and the presence of mtDNA CD, occurring both in lung cancer patients – mtDNA CD was found in 73.3% of the smokers and 23.1% of nonsmokers – and in individuals without lung cancer – mtDNA CD was present in 44.4% of the smokers and in none of the nonsmokers – suggests that environmental factors (cigarette smoke in this case) may induce the mtDNA CD in "normal" tissue (Dai et al. 2006). The relation between smoking and mtDNA lesions had already been observed by Ballinger et al. (1996) who analyzed the bronchoalveolar lavage from smokers and nonsmokers. The authors observed that smokers presented significantly higher levels of nuclear and mtDNA damage than nonsmokers. Whenever a cancer develops in those cells harboring the mtDNA CD, all cancer cells should present this lesion, a phenomenon which is called "primary" oxyphilia (see below).

Mitochondria and Oncocytic Tumors of the Parathyroid

The parathyroid glands have the peculiarity to present normally a variable percentage of oncocytic cells. Müller-Höcker et al. (1996) evaluated the status of mitochondria in normal parathyroid glands and showed that defects of the respiratory chain

are present in normal parathyroids, most probably during cell ageing, and especially in the oncocytic cells. The high predominance of respiratory chain defects in oncocytic cells and their random distribution favor mutations of mtDNA as a possible cause of oncocytic cell transformation (Muller-Hocker et al. 1996). The rarity of consistent molecular genetic abnormalities regarding the presence of mtDNA CD and point mutations led Müller-Höcker et al. (1996) to conclude that mutations of mtDNA in the parathyroids are apparently different from those in other ageing tissues.

Parathyroid adenomas composed predominantly (more than 90%) or exclusively of oncocytic cells are uncommon; according to Apel and Asa (2002) they constitute 4.4–8.4% of all parathyroid adenomas. It was advanced that oncocytic parathyroid adenomas and parathyroid adenomas with a prominent oncocytic component, although rare, tend to be larger and associated with minimal hyperparathyroidism (Giorgadze et al. 2004). The rare oncocytic parathyroid carcinoma, show similar prognostic characteristics as chief cell carcinomas and are associated with recurrent disease and death in around 50% of the cases (Erickson et al. 2005).

Müller-Höcker (1992), described random cytochrome-C-oxidase deficiency in oncocytic cell adenomas, whereas no abnormalities were detected in other enzymes of the respiratory chain (succinate dehydrogenase and ATP synthase). Genetic studies are necessary to see if these mitochondrial protein alterations reflect mtDNA alterations.

Familial Oncocytomas

A gene predisposing to familial oncocytic thyroid tumors was mapped to chromosome 19p13.2 although the identity of such gene remains unknown (Canzian et al. 1998). Recently, a novel mitochondrial-related gene, *GRIM-19*, has been identified. The protein GRIM-19 is a cell death regulatory protein that promotes apoptosis, negatively regulates cell growth, and is also involved in mitochondrial metabolism (Angell et al. 2000). *GRIM-19* location was assigned to 19p13.2, making it a good candidate for the gene predisposing to familial oncocytic thyroid tumors reported by Canzian et al. (1998). Máximo et al. (2005a) have searched for *GRIM-19* mutations in a series of 52 thyroid tumors. Sequencing of the five exons of *GRIM-19* in the 20 apparently sporadic oncocytic cell tumors disclosed the existence of four mutations: three somatic missense mutations and a germline mutation detected in a oncocytic cell papillary carcinoma arising in a thyroid with multiple oncocytic cell benign nodules (Maximo et al. 2005a). No mutations were detected in any of the 20 cases of non-oncocytic follicular and papillary carcinomas, nor in any of the 96 blood donor samples (Maximo et al. 2005a). The *GRIM-19* mutations detected are the first nuclear gene mutations specific to oncocytic cell tumors to be reported to date (Maximo et al. 2005a).

One of the sporadic oncocytic cell papillary carcinomas, positive for *GRIM-19* mutation, also harbored a RET/PTC-1 rearrangement, suggesting that *GRIM-19*

mutation may serve as a predisposing alteration for the occurrence of tumors with oncocytic cells, and other alterations, such as RET/PTC rearrangements or *B-RAF* mutations, may be necessary for the acquisition of the malignant phenotype, as in non-oncocytic cell papillary carcinomas (Maximo et al. 2005a). Bonora et al. (2006a) described a family with thyroid oncocytomas in which there was linkage with a polymorphism in the gene encoding the translocase of the inner mitochondrial membrane 44 (*TIMM44*).

In addition to the mutations identified in oncocytic thyroid tumors, the status of *GRIM-19* was also evaluated in RCC. Alchanati et al. (2006) observed, using mass spectrometry, that the expression of *GRIM-19* is lost, or severely depressed, in several primary RCC, indicating that *GRIM-19* may play a role in RCC.

The timing of occurrence of the somatic mtDNA mutations (or the environmental aggression) in relation to the initiation of the neoplastic process may lead to "primary" or "secondary" oncocytic cell tumors. If the factor that leads to the increased amount of mitochondria occurs in an already initiated neoplastic cell, the resulting tumor will most likely display the architectural, biochemical and clinicopathological features of the corresponding conventional tumor (papillary, follicular or medullary) and will progressively acquire oncocytic features ("secondary" oxyphilia). If the mitochondrial hit occurs in a normal cell that will later on develop towards a neoplasm through a series of mutations, the final product will be a "primary" oncocytic cell tumor (Fig. 5). Degenerative diseases due to mtDNA mutations (MITOMAP) can present a familial pattern, but the same is not true for oncocytic tumors. None of the mitochondrial syndromes on record have been associated with a higher prevalence of oncocytic tumors.

The same seems to be not true concerning mutations in nuclear genes encoding mitochondrial proteins. Two ubiquitously expressed mitochondrial enzymes – succinate dehydrogenase (SDH) and fumarate hydratase (FH, also called fumarase) –catalyse sequential steps in the Krebs tricarboxylic-acid cycle. Inherited

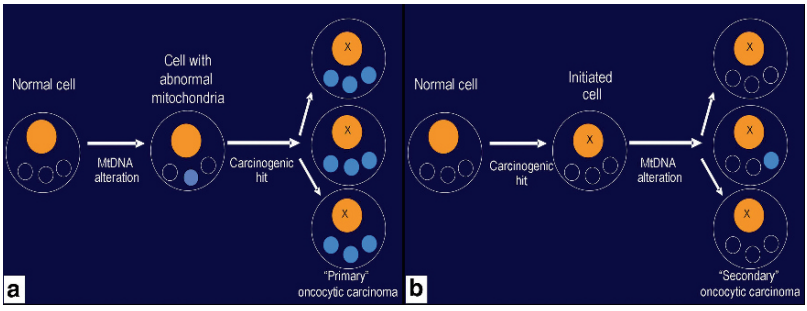

Fig. 5 Diagram exemplifying the occurrence of a primary (**a**) and secondary (**b**) oncocytic carcinoma. Empty circles represent normal mitochondria, blue-filled circles represent abnormal mitochondria and orange-filled circles represent nuclei. An "X" in the nuclei represents a tumorigenic alteration in the nuclear DNA (adapted from Sobrinho-Simões et al., Int J Surg Pathol 13:29–35, 2005) (See Color Insert)

heterozygous mutations in *SDHB*, *SDHC*, or *SDHD* (three genes that encode three of the four homonymous subunits of SDH), as well as in *FH*, cause predisposition to two types of inherited neoplastic syndromes that do not share any component tumors – hereditary paragangliomas/pheochromocytomas and hereditary uterine leiomyomas/RCC, respectively (Gottlieb and Tomlinson 2005). Interestingly, homozygous mutations both in the gene encoding the fourth subunit of SDH – *SDHA* – and in *FH* result in severe neurological impairment (Eng et al. 2003).

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References

Alchanati, I., Nallar, S. C., Sun, P., Gao, L., Hu, J., Stein, A., Yakirevich, E., Konforty, D., Alroy, I., Zhao, X., Reddy, S. P., Resnick, M. B., and Kalvakolanu, D. V. 2006. A proteomic analysis reveals the loss of expression of the cell death regulatory gene GRIM-19 in human renal cell carcinomas. Oncogene 25:7138–7147.

Angell, J. E., Lindner, D. J., Shapiro, P. S., Hofmann, E. R., and Kalvakolanu, D. V. 2000. Identification of GRIM-19, a novel cell death-regulatory gene induced by the interferon-beta and retinoic acid combination, using a genetic approach. J. Biol. Chem. 275:33416–33426.

Apel R. L. and Asa S. L. 2002. The parathyroid glands. In Endocrine Pathology, V. LiVolsi and S. L. Asa (eds.), pp. 103–137. Philadelphia, PA: Churchill Livingstone.

Attardi, G., Yoneda, M., and Chomyn, A. 1995. Complementation and segregation behavior of disease-causing mitochondrial DNA mutations in cellular model systems. Biochim Biophys Acta 1271:241–248.

Ballinger, S. W., Bouder, T. G., Davies, G. S., Judice, S. A., Nicklas, J. A., and Albertini, R. J. 1996. Mitochondrial genome damage associated with cigarette smoking. Cancer Res. 56:5692–5697.

Baris, O., Savagner, F., Nasser, V., Loriod, B., Granjeaud, S., Guyetant, S., Franc, B., Rodien, P., Rohmer, V., Bertucci, F., Birnbaum, D., Malthiery, Y., Reynier, P., and Houlgatte, R. 2004. Transcriptional profiling reveals coordinated up-regulation of oxidative metabolism genes in thyroid oncocytic tumors. J. Clin. Endocrinol. Metab. 89:994–1005.

Bonora, E., Evangelisti, C., Bonichon, F., Tallini, G., and Romeo, G. 2006a. Novel germline variants identified in the inner mitochondrial membrane transporter TIMM44 and their role in predisposition to oncocytic thyroid carcinomas. Br. J. Cancer 95:1529–1536.

Bonora, E., Porcelli, A. M., Gasparre, G., Biondi, A., Ghelli, A., Carelli, V., Baracca, A., Tallini, G., Martinuzzi, A., Lenaz, G., Rugolo, M., and Romeo, G. 2006b. Defective oxidative phosphorylation in thyroid oncocytic carcinoma is associated with pathogenic mitochondrial DNA mutations affecting complexes I and III. Cancer Res. 66:6087–6096.

Brooks, J. D., Marshall, F. F., Isaacs, W. B., and Johns, D. R. 1999. Absence of HinfI Restriction Abnormalities in Renal Oncocytoma Mitochondrial DNA. Mol. Urol. 3:1–3.

Canzian, F., Amati, P., Harach, H. R., Kraimps, J. L., Lesueur, F., Barbier, J., Levillain, P., Romeo, G., and Bonneau, D. 1998. A gene predisposing to familial thyroid tumors with cell oxyphilia maps to chromosome 19p13.2. Am. J. Hum. Genet. 63:1743–1748.

Czarnecka, A. M., Golik, P., and Bartnik, E. 2006. Mitochondrial DNA mutations in human neoplasia. J. Appl.Genet. 47:67–78.

Dai, J. G., Xiao, Y. B., Min, J. X., Zhang, G. Q., Yao, K., and Zhou, R. J. 2006. Mitochondrial DNA 4977 BP deletion mutations in lung carcinoma. Ind. J. Cancer 43:20–25.

DeLellis, R. A., Lloyd, R. V., Heitz, P. U., and Eng, C. (eds.) 2004. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Endocrine Organs. Lyon: IARC press.

Eng, C., Kiuru, M., Fernandez, M. J., and Aaltonen, L. A. 2003. A role for mitochondrial enzymes in inherited neoplasia and beyond. Nat. Rev. Cancer. 3:193–202.

Erickson, L. A., Jin, L., Papotti, M., and Lloyd, R. V. 2005. Oxyphil parathyroid carcinomas: a clinicopathologic and immunohistochemical study of 10 cases. Am. J. Surg. Pathol. 26:344–349.

Farrand, K., Delahunt, B., Wang, X. L., McIver, B., Hay, I. D., Goellner, J. R., Eberhardt, N. L., and Grebe, S. K. 2002. High resolution loss of heterozygosity mapping of 17p13 in thyroid cancer: Hurthle cell carcinomas exhibit a small 411-kilobase common region of allelic imbalance, probably containing a novel tumor suppressor gene. J. Clin. Endocrinol. Metab. 87:4715–4721.

Frisk, T., Kytola, S., Wallin, G., Zedenius, J., and Larsson, C. 1999. Low frequency of numerical chromosomal aberrations in follicular thyroid tumors detected by comparative genomic hybridization. Genes Chromosomes Cancer 25:349–353.

Gasparre, G., Porcelli, A. M., Bonora, E., Pennisi, L. F., Toller, M., Iommarini, L., Ghelli, A., Moretti, M., Betts, C. M., Martinelli, G. M., Ceroni, A. R., Curcio, F., Carelli, V., Rugolo, M., Tallini, G., and Romeo, G. 2007. Disruptive mitochondrial DNA mutations in complex I subunits are markers of oncocytic phenotype in thyroid tumors. Cancer Res. 104:9001–9006.

Giorgadze, T., Stratton, B., Baloch, Z. W., and LiVolsi, V. A. 2004. Oncocytic parathyroid adenoma: problem in cytological diagnosis. Diagn. Mol. Pathol. 31:276–280.

Gottlieb, E. and Tomlinson, I. P. 2005. Mitochondrial tumour suppressors: a genetic and biochemical update. Nat. Rev. Cancer 5:857–866.

Hamperl, H. 1962. Benign and malignant oncocytoma. Cancer. 15:1019–1027.

Heddi, A., Faure-Vigny, H., Wallace, D. C., and Stepien, G. 1996. Coordinate expression of nuclear and mitochondrial genes involved in energy production in carcinoma and oncocytoma. Biochim. Biophys. Acta. 1316:203–209.

Jacques, C., Baris, O., Prunier-Mirebeau, D., Savagner, F., Rodien, P., Rohmer, V., Franc, B., Guyetant, S., Malthiery, Y., and Reynier, P. 2005. Two-step differential expression analysis reveals a new set of genes involved in thyroid oncocytic tumors. J. Clin. Endocrinol. Metab. 90:2314–2320.

Kepes, J. J. 1983. Oncocytic transformation of choroid plexus epithelium. Acta Neuropathol. 63:145–148.

Lewis, P. D., Baxter, P., Paul Griffiths, A., Parry, J. M., and Skibinski, D. O. 2000. Detection of damage to the mitochondrial genome in the oncocytic cells of Warthin's tumour. J. Pathol. 191:274–281.

Lewis, P. D., Fradley, S. R., Griffiths, J. R., Baxter, P. W., and Parry, J. M. 2002. Mitochondrial DNA mutations in the parotid gland of cigarette smokers and non-smokers. Mutat. Res. 518:47–54.

Lindal, S., Lund, I., Torbergsen, T., Aasly, J., Mellgren, S. I., Borud, O., and Monstad, P. 1992. Mitochondrial diseases and myopathies: a series of muscle biopsy specimens with ultrastructural changes in the mitochondria. Ultrastruct. Pathol. 16:263–275.

Maximo, V. and Sobrinho-Simoes, M. 2000a. Hurthle cell tumours of the thyroid. A review with emphasis on mitochondrial abnormalities with clinical relevance. Virchows Arch. 437:107–115.

Maximo, V. and Sobrinho-Simoes, M. 2000b. Mitochondrial DNA 'common' deletion in Hurthle cell lesions of the thyroid. J. Pathol. 192:561–562.

Maximo, V., Sores, P., Rocha, A. S., and Sobrinho-Simoes, M. 1998. The common deletion of mitochondrial DNA is found in goiters and thyroid tumors with and without oxyphil cell change. Ultrastruct. Pathol. 22:271–273.

Maximo, V., Soares, P., Lima, J., Cameselle-Teijeiro, J., and Sobrinho-Simoes, M. 2002. Mitochondrial DNA somatic mutations (point mutations and large deletions) and mitochondrial DNA variants in human thyroid pathology: a study with emphasis on Hurthle cell tumors. Am. J. Pathol. 160:1857–1865.

Maximo, V., Preto, A., Crespo, A., Rocha, A. S., Machado, J. C., Soares, P., and Sobrinho-Simoes, M. 2004. Core I gene is overexpressed in Hurthle and non-Hurthle cell microfollicular adenomas and follicular carcinomas of the thyroid. BMC Cancer 25:4–12.

Maximo, V., Botelho, T., Capela, J., Soares, P., Lima, J., Taveira, A., Amaro, T., Barbosa, A. P., Preto, A., Harach, H. R., Williams, D., and Sobrinho-Simoes, M. 2005a. Somatic and germline mutation in GRIM-19, a dual function gene involved in mitochondrial metabolism and cell death, is linked to mitochondrion-rich (Hurthle cell) tumours of the thyroid. Br J Cancer 92:1892–1898.

Maximo, V., Lima, J., Soares, P., Botelho, T., Gomes, L., and Sobrinho-Simoes, M. 2005b. Mitochondrial D-Loop instability in thyroid tumours is not a marker of malignancy. Mitochondrion 5:333–340.

MITOMAP: A Human Mitochondrial Genome Database, 2007. http://www.mitomap.org.

Muller-Hocker, J. 1992. Random cytochrome-C-oxidase deficiency of oxyphil cell nodules in the parathyroid gland. A mitochondrial cytopathy related to cell ageing? Pathol. Res. Pract. 188:701–706.

Muller-Hocker, J., Aust, D., Napiwotsky, J., Münscher, C., Link, T. A., Seibel, P., Schneeweiss, S. G., and Kadenbach, B. 1996. Defects of the respiratory chain in oxyphil and chief cells of the normal parathyroid and in hyperfunction. Hum. Pathol. 27:532–541.

Nesland, J. M., Sobrinho-Simoes, M. A., Holm, R., Sambade, M. C., and Johannessen, J. V. 1985. Hurthle-cell lesions of the thyroid: a combined study using transmission electron microscopy, scanning electron microscopy, and immunocytochemistry. Ultrastruct. Pathol. 8:269–290.

Ohama, E. and Ikuta, F. 1987. Involvement of choroid plexus in mitochondrial encephalomyopathy (MELAS). Acta Neuropathol. 75:1–7.

Pavlovich, C. P., Walther, M. M., Eyler, R. A., Hewitt, S. M., Zbar, B., Linehan, W. M., and Merino, M. J. 2002. Renal tumors in the Birt-Hogg-Dubé syndrome. Am. J. Surg. Pathol. 26:1542–1552.

Sanchez-Cespedes, M., Parrella, P., Nomoto, S., Cohen, D., Xiao, Y., Esteller, M., Jeronimo, C., Jordan, R. C., Nicol, T., Koch, W. M., Schoenberg, M., Mazzarelli, P., Fazio, V. M., and Sidransky, D. 2001. Identification of a mononucleotide repeat as a major target for mitochondrial DNA alterations in human tumors. Cancer Res. 61:7015–7019.

Savagner, F., Franc, B., Guyetant, S., Rodien, P., Reynier, P., and Malthiery, Y. 2001. Defective mitochondrial ATP synthesis in oxyphilic thyroid tumors. J. Clin. Endocrinol. Metab. 86:4920–4925.

Sengers, R. C., Stadhouders, A. M., and Trijbels, J. M. 1984. Mitochondrial myopathies. Clinical, morphological and biochemical aspects. Eur. J. Pediatr. 141:192–207.

Simonnet, H., Alazard, N., Gallou, C., Béroud, C., Demont, J., Bouvier, R., Schägger, H., and Godinot, C. 2002. Low mitochondrial respiratory chain content correlates with tumor aggressiveness in renal cell carcinoma. Carcinogenesis 23:759–768.

Simonnet, H., Demont, J., Pfeiffer, K., Guenaneche, L., Bouvier, R., Brandt, U., Schagger, H., and Godinot, C. 2003. Mitochondrial complex I is deficient in renal oncocytomas. Carcinogenesis 24:1461–1466.

Sobrinho-Simoes, M., Maximo, V., Castro, I. V., Fonseca, E., Soares, P., Garcia-Rostan, G., and Oliveira, M. C. 2005. Hurthle (oncocytic) cell tumors of thyroid: etiopathogenesis, diagnosis and clinical significance. Int. J. Surg. Pathol. 13:29–35.

Sobrinho-Simoes, M. A., Nesland, J. M., Holm, R., Sambade, M. C., and Johannessen, J. V. 1985. Hurthle cell and mitochondrion-rich papillary carcinomas of the thyroid gland: an ultrastructural and immunocytochemical study. Ultrastruct. Pathol. 8:131–142.

Tallini, G., Hsueh, A., Liu, S., Garcia-Rostan, G., Speicher, M. S., and Ward, D. C. 1999. Frequent chromosomal DNA unbalance in thyroid oncocytic (Hürthle cell) neoplasms detected by comparative genomic hybridization. Lab. Invest. 79:547–555.

Tallini, G., Ladanyi, M., Rosai, J., and Jhanwar, S. C. 1994. Analysis of nuclear and mitochondrial DNA alterations in thyroid and renal oncocytic tumors. Cytoget. Cell Genet. 66:253–259.

Tanji, K., Schon, E. A., DiMauro, S., and Bonilla, E. 2000. Kearns-sayre syndrome: oncocytic transformation of choroid plexus epithelium. J. Neurol. Sci. 178:29–36.

Taylor, R. W. and Turnbull, D. M. 2005. Mitochondrial DNA mutations in human disease. Nat. Rev. Genet. 6:389–402.

Wada, N., Duh, Q. Y., Miura, D., Brunaud, L., Wong, M. G., and Clark, O. H. 2002. Chromosomal aberrations by comparative genomic hybridization in hürthle cell thyroid carcinomas are associated with tumor recurrence. J. Clin. Endocrinol. Metab. 87:4595–4601.

Welter, C., Kovacs, G., Seitz, G., and Blin, N. 1989. Alteration of mitochondrial DNA in human oncocytomas. Genes Chromosomes Cancer 1:79–82.

Zambrano, N. R., Lubensky, I. A., Merino, M. J., Linehan, W. M., and Walther, M. M. 1999. Histopathology and molecular genetics of renal tumors toward unification of a classification system. J. Urol. 162:1246–1258.

Mitochondria as Targets for Cancer Therapy

Stephen J. Ralph and Jiri Neuzil

Introduction: Molecularly Targeted Precision Heralds New-Age Cancer Therapy

Treatments that selectively target and kill cancer cells without affecting normal cells would represent the panacea for cancer. Gleevec (Imatinib) treatment of chronic myelogenous leukaemia (CML) provided the first example that such a feat was indeed possible by targeting the Bcr-Abl tyrosine kinase fusion protein specific to the survival of this type of cancer (O'Hare et al. 2006). Together with the development of the monoclonal antibody Herceptin (Trastuzimab), which inhibits the HER2 (erbB2) receptor on breast cancer cells (Hsieh and Moasser 2007), the drugs Gleevec and Herceptin have confirmed that the age of molecularly targeted cancer therapeutics has finally arrived.

We are now seeing another exciting time in cancer therapy with a range of compounds in development that selectively target mitochondria in cancer cells. The mitochondria acting as the powerhouses producing cellular energy are potential "powder kegs," which when ignited can culminate in the death of malignant cells. These novel drugs offer substantial promise as potent and effective clinical treatments with minimal side-effects compared with current chemotherapeutic approaches. Although still in the early phase of human clinical trials, an in-depth analysis and understanding at the molecular level of how mitochondrial targeted anticancer drugs selectively kill cancer cells supports such claims and their future potential.

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Targeting Differences in Energy Metabolism between Cancer and Normal Cell Mitochondria to Selectively Destroy Cancer Cells

Understanding the molecular basis of metabolic changes that distinguish cancer cells from normal cells assists identification of sensitive targets with precise accuracy and provides increasingly more effective therapies that selectively kill cancer cells with little or no harm to the normal cells. Hence, this review focuses on the mechanisms whereby metabolic changes affecting mitochondrial function inside cancer cells provide these cells with distinctive properties and survival advantages worth targeting. Particular focus relates to the emerging theme that mitochondria, as key sensors for activating the destructive suicide signal, have this function suppressed in cancer cells. Consequently, the main emphasis in this chapter is on mitochondrial function and reactivation of the death signal pathway(s) in cancers and particularly apoptosis. The more recent concept of suppression of autophagy and the theory that damaged mitochondria remain, promoting the onset of cell malignancy, is not covered.

The chapter also reviews many different types of anticancer therapeutics currently under development that target mitochondrial function with the aim of inducing cancer cell death. To simplify the generic naming for this class of compounds, we have coined the abbreviation "mitocans" for the more verbose terminology "mitochondrially targeted anticancer compounds" encompassing the expanding list of different subgroups. We describe the growing number of mitocans, their mechanisms for targeting mitochondria leading to activation of cancer cell death pathways, and discuss reports from clinical studies that are showing promise.

One of the essential aspects of mitocan therapies that target cancer and not normal cells relates to the differences in properties of mitochondria from these two cell types. The general metabolic state of cancer cells was reported early on by Warburg et al. (1930) to differ significantly from that of normal cells, with cancer cells exploiting higher levels of glycolysis for their growth and survival whether they grew under anaerobic or aerobic conditions. In general, mitochondrial function including respiration is greatly reduced in cancer cells as they grow in progressively hypoxic environments during the development and progression of the tumour mass. Consequently, tumour microenvironments differ greatly from that of normal tissues, particularly in their interior vasculature and regions of hypoxia (reviewed in Brown and Giaccia 1998; Knowles and Harris 2001; Vaupel and Mayer 2007). This results in metabolic modifications reflecting the growth of tumour cells under hypoxia, including changes in signalling pathways and transcription factors inside cancer cells that lead to significant alterations in gene expression. The major changes in energy metabolism affecting mitochondrial function in cancer cells are the subject of the following sections.

Hypoxia during Cancer Progression Leads to HIF-Induced Changes in Energy Flow to Mitochondria

After genetic mutation in cell growth regulatory genes, perhaps the next most significant contributing factor to cancer cell development is the dysregulation in the supply of oxygen available to a tumour. Thus, during the emergence of a cancer, the cells in the tumour microenvironment undergo intermittent periods of oxygen deprivation (Brown and Giaccia 1998). This likely occurs as a result of rapid cell growth shifting cells from a state of sufficient nutrient and oxygen supply from the nearby vasculature to one of competition, relative starvation and hypoxia as the tumour volume expands. In response to the hypoxic stress, the tumour cells, particularly in more remote regions distant from the prevailing blood supply of oxygen, undergo a selection process that involves the activation of a transcription factor called hypoxia-inducible factor (HIF).

HIF is a master oxygen sensor and regulator of energy metabolism such that when oxygen is available, the HIFα subunit has a short half life because it becomes modified by oxygen-dependent posttranslational hydroxylation, mediated by prolyl hydroxylases (PHDs 1–3). Prolyl hydroxylation of HIF acts as a marker for its ubiquitination by the von Hippel Lindau (VHL) E3 ligase, which targets HIF to the proteasomal complex for degradation (reviewed in Kim et al. 2007; Brahimi-Horn et al. 2007). Hence, HIF is not usually activated in cells under normoxic conditions.

By contrast, when cells become hypoxic, the α subunit is no longer turned over rapidly and instead it binds the HIFβ subunit to produce the HIFα/β heterodimer, an active transcription factor complex (Brahimi-Horn et al. 2007) inducing expression of numerous genes (reviewed in Semenza 2007), including key enzymes that increase the glycolytic pathway flux. HIF also induces vascular endothelial growth factor (VEGF) expression, resulting in formation of new blood vessels within the tumour vasculature, which helps restore the supply of nutrients and oxygen (Brahimi-Horn et al. 2007).

Among the many genes induced by HIF activation three are particularly important to the altered state of energy metabolism in cancer cells, severely limiting mitochondrial function. The first encodes hexokinase II (HK-II) that becomes overexpressed and bound to the mitochondrial outer membrane (MOM) via the porin-like protein voltage-dependent anion channel (VDAC) protein (Fig. 1). Together with the adenine nucleotide transporter (ANT), these proteins form a channel that transports ATP made by the mitochondrial inner membrane (MIM)-located ATP synthase directly to active sites on HK-II. The higher levels of HK-II bind both ATP and incoming glucose, producing increased glucose-6-phosphate levels in the cytosol. Bustamante et al. (1981) established a correlation between the growth rate of tumours and their mitochondrial associated HK activity. Among 12 different tumours tested, only those with rapid growth rates showed mitochondrial associated HK activity. In addition, according to Koobs (1972), mitochondrial-bound HK limits respiration when tumour cells are utilizing glucose as their energy source (a situation known as the Crabtree effect). Even though large amounts of ADP are made in this situation, the continuous phosphorylation of glucose by ATP (through

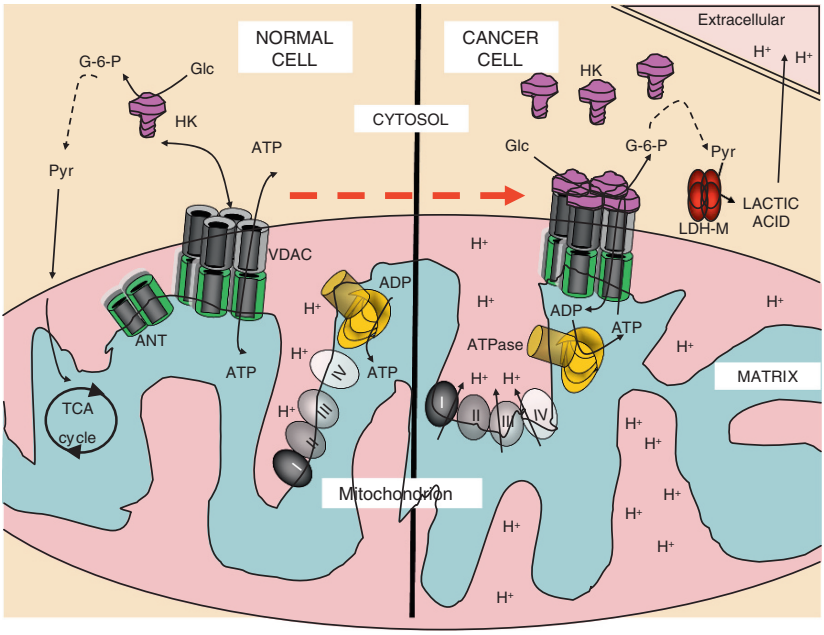

Fig. 1 Comparison of mitochondrial metabolism between a normal vs. a cancer cell. In normal cells, pyruvate formed from glycolysis is transported into the mitochondria to be converted for entry into the TCA cycle. In cancer cells, much greater levels of hexokinase exist bound to mitochondria, promoting greater glycolysis and preventing ion channel flow via VDAC. In cancer cells, pyruvate does not enter the mitochondria as it mostly converts to lactic acid in the cytosol that in turn, ends up acidifying the extracellular milieu. Cancer mitochondria show a greater transmembrane potential across the MIM, reduced OXPHOS and flux along the electron transport chain

mitochondria-bound HK) also reduces phosphate levels available for oxidative phosphorylation (OXPHOS) and will prevent the tumour cell mitochondria from entering into the maximum rate of state 3 respiration (reviewed in Baggetto 1992). HK binding to VDAC on the mitochondria appears to restrict channel conductance, including the entry of Ca^{2+} ions from the cytosol required to induce apoptosis. Hence, HK binding stabilizes tumour cell mitochondria, suppressing apoptosis and promoting cancer cell survival (Mathupala et al. 2006).

The second HIF-activated gene encodes a distinct lactate dehydrogenase isoform (LDH-M) while the third gene encodes pyruvate dehydrogenase kinase (PDK-1). These two genes are important because they cause significant changes to metabolism of pyruvate following glycolysis. In nonmalignant cells metabolising under normoxia, much of the pyruvate is transported into the mitochondria for pyruvate dehydrogenase production of acetyl-CoA for entry into the TCA cycle. In cancer cells, after HIF activation, the situation is very different because LDH-M and PDK-1 expression and activity are greatly elevated and these enzymes control the flow of pyruvate from glycolysis by effectively restricting pyruvate from entering into mitochondria, instead re-directing pyruvate in the cytosol to produce high levels of lactic acid.

LDH exists in two different isoforms, each encoded by separate genes; LDH-L is a low-activity isoform and LDH-M is the more active isoform that catalyses the reduction of pyruvate with NADH to produce lactic acid and NAD^+ (Firth et al. 1995; Koukorakis et al. 2005). The LDH enzyme exists as tetramers containing different ratios of the two isoforms and in cancer cells the predominant species is a tetramer of LDH-M with the highest catalytic activity. The LDH-M tetramer (also known as LDH5 or LDHA) and the Keq of pyruvate to lactate reaction greatly enhances lactic acid production, particularly with increasing glycolysis and high levels of NADH (Brooks 1998, 2000; Brooks et al. 1999a, b). In head and neck carcinomas and many other human cancers, excessive tumour lactate levels are significantly correlated with the likelihood of metastases and poor clinical outcome (34). Furthermore, high levels of the LDH5 enzyme isoform have also been associated with advanced and aggressive forms of many different types of cancers (Koukourakis et al. 2005; Walenta and Mueller-Klieser 2004).

PDK-1 directly modifies cancer cell pyruvate metabolism by phosphorylating PDH, inhibiting its activity, with the net effect that pyruvate becomes more available in the cytosol as it is no longer metabolised by PDH to acetyl coA in mitochondria (Papandreou et al. 2006; Kim et al. 2007; Roche and Hiromasa 2007). The result of inhibiting PDH and pyruvate metabolism via the TCA cycle, favouring pyruvate conversion to lactic acid, is that there is a significant reduction in the mitochondrial production and availability of substrates like NADH and succinate required for the respiratory pathway.

The major modifications induced by HIF, acting to increase PDK and LDH activity and their effects on pyruvate metabolism, as well as the shift to greater glycolysis, are also associated with other metabolic changes. Thus, cancer cells exhibit a shift in aldehyde dehydrogenase isoforms to those with reduced affinity for the substrate acetaldehyde, leading to increased acetaldehyde (Baggetto 1992). The increase in acetaldehyde turns the pyruvate dehydrogenase (PDH) complex into a nonoxidative decarboxylase converting pyruvate to acetaldehyde, a highly reactive species. Within the PDH complex, acetaldehyde also reacts with decarboxylated pyruvate (as a hydroxyethyl-thiamine-pyrophosphate-enzyme complex) to form acetoin, a neutral compound not normally present in mammalian cells (Baggetto and Lehninger 1987). At this point, with its ensuing build up, acetoin becomes a competitive inhibitor, also preventing the PDH complex from using pyruvate in tumours. In addition, acetoin build up in tumour cells appears to directly inhibit succinate oxidation via complex II of the respiratory chain, further adding to the inhibition of mitochondrial respiratory function in tumour cells (Baggetto and Testa-Parussini 1990). The low residual pyruvate (5–15%, reviewed in Baggetto 1992) not converted to lactic acid is most probably transported into the mitochondria to produce acetoin. Nevertheless, many different factors contribute to prevent pyruvate entering into the tricarboxylic acid (TCA) cycle in mitochondria of cancer cells. The net outcomes from HIF activation and acetoin production are that mitochondrial oxygen consumption and ATP production are inhibited and cancer cells mainly survive on glycolysis.

Pseudohypoxia

Studies of particular types of human neuronal malignancies, such as pheochromocytomas, paragangliomas, leiomyomas and renal-cell carcinomas has provided insight into one protective device, whereby cancer cells promote their own survival. This process has become known as "pseudohypoxia" as distinct from the usual hypoxic development of cancers described in the previous section. This is because these cancers contain mutations that make the cells behave as if they were hypoxic, even when normal levels of oxygen are present. Thus, in the pseudohypoxic cancers, somatic mutations occur in either the succinate dehydrogenase (SDH) or fumarate hydratase (FH, or fumarase), enzymes that are part of the tricarboxylic acid (TCA) cycle linking glucose metabolism in the cytosol and OXPHOS in mitochondria. The *SDHA* gene encodes the enzymatic subunit SDHA, located on the matrix side of the MIM, associated with three other subunits (SDHB, SDHC and SDHD) of the ubiquinone oxidoreductase complex II. Inherited or somatic mutations in *SDHB*, *SDHC* or *SDHD* genes are associated with phaeochromocytomas or paragangliomas, whereas those in the *FH* gene are associated with leiomyomas, leiomyosarcomas or renal cell cancers (reviewed in Gottlieb and Tomlinson 2005).

As a consequence of mutations in the genes encoding SDH or FH, a build up in the levels of succinate or fumarate increases levels of both intermediates of the TCA cycle. The two metabolites feed back to inhibit prolyl hydroxylase (PHD3/ENGL3) (Selak et al. 2005; Lu et al. 2005; Pollard et al. 2005; King et al. 2006; Koivunen et al. 2007; MacKenzie et al. 2007). In this event, the transcription factor HIF-1α is no longer modified by PHDs to facilitate its ubiquitination and degradation. This situation resembles the same state found in hypoxia (and hence the "pseudohypoxia") in that levels of activated HIF-1α become elevated, despite the continued presence of oxygen. The downstream effects of higher HIF-1α levels are to promote the malignancy of the cells as described for the hypoxic situation in the previous section, i.e., high levels of aerobic glycolysis and reduced mitochondrial respiration.

Other situations also occur in cancers where a pseudohypoxic condition is established. One example is the von Hippel Lindau (VHL) mutation, where the VHL tumour suppressor protein is absent. As this gene encodes the E3 ligase required for ubiquitinating HIF-1α, when it is missing, HIF-1α becomes stabilized even in nonhypoxic cells, promoting the relevant metabolic changes supporting the malignant phenotype (reviewed in Kim et al. 2007).

The 2-oxoacids, pyruvate and oxaloacetate, can also feed back to inhibit prolyl hydroxylase 1 and 2 (HPH or PHD 1, Dalgard et al. 2004). However, these metabolites work by a different mechanism to that of succinate and fumarate described earlier (Lu et al. 2005), possibly by targeting distinct PHD isoforms. Even in the presence of oxygen, most cancer cells accumulate high levels of pyruvate and lactate (Warburg 1930), as described earlier in this section. Therefore, given the significant role of HIF-1, accumulation of pyruvate and oxaloacetate as a result of increased glycolysis could contribute to cancer cell development by

providing a feedback pathway promoting increased activation of HIF-1, independent of the levels of oxygenation.

Proton Flux Regulation and Potential Difference across the Mitochondrial Inner Membrane of Cancer Cells

Another commonly observable difference in cancer cell compared with normal cell mitochondria is the greater mitochondrial inner trans-membrane potential ($\Delta\Psi_{m,i}$) in cancer cells. For example, as a result of the metabolic changes occurring inside cancer cells and their mitochondria, the $\Delta\Psi_{m,i}$ is increased to greater negative values (-150 to -170 mV, negative inside the matrix) in carcinoma cells (Summerhayes et al. 1982; Lampidis et al. 1983; Chen 1988; Modica-Napolitano and Aprille 1987; 2001), with ~60 mV difference across the MIM. Many proposals have been made to explain the reasons for this difference in membrane potential. At the molecular level, these include differences in mitochondrial respiratory enzyme complexes, electron carriers, ATPase, ANT and/or changes in membrane lipid metabolism. Other proposals for the increased mitochondrial membrane potential in cancer cells include altered electron transfer activity, proton translocation and utilization, or conductance. For example, mitochondria isolated from hepatocellular carcinomas display reduced uncoupler-stimulated ATP hydrolysis, decreased rates of respiration-linked ATP synthesis and reduced phosphorylating capacity compared with normal liver cells (Pedersen et al. 1974; Capuano et al. 1996, 1997; Cuezva et al. 1997).

Marked changes in enzyme function, particularly in the ATPase, have been shown to occur in cancer cell mitochondria. Thus, preparations of ATPase isolated from carcinomas show reduced maximal velocity, decreased immunodetectable levels of the β subunit of the F1 component of mitochondrial ATPase and/or overexpression of the ATPase inhibitor protein (IF1) (Pedersen et al. 1974; Capuano et al. 1996, 1997; Cuezva et al. 1997; reviewed in Modica-Napolitano and Singh 2002). A reduced ability to use the proton gradient to make ATP, with a resulting build up in the protons within the MIM would account for the greater $\Delta\Psi_{m,i}$ existing in tumour mitochondria.

Another possibility that may account for greater $\Delta\Psi_{m,i}$ in cancer cells is that acetoin undergoes an ATP-dependent reaction, almost doubling the reaction rate to produce citrate in tumour cells (Baggetto and Lehninger 1987; Baggetto and Testa-Parussini 1990), which is then exported by the tricarboxylate or citrate carrier (CIC) to the cytosol, where it is cleaved to oxaloacetate and acetyl-coA. The net effect is the provision of a high level of cytoplasmic acetyl-coA precursor for sterol biosynthesis, particularly helping to promote the already elevated cancer cell production of cholesterol (Baggetto and Testa-Parussini 1990). The resulting build-up of cholesterol in the inner MIM reduces several fold their passive proton permeability, helping to establish the greater transmembrane potential in cancer cells (Baggetto and Testa-Parussini 1990; Baggetto et al. 1992).

Proton Flux across the Plasma Membrane Enables Cancer Cells to Be Selective Targets for Anticancer Drugs

The enhanced glycolytic activity due to very high energetic demand increases cytoplasmic levels of lactic acid production in cancer cells. To maintain the neutral pH of the cytosol, these cells activate plasma membrane proton pumps causing extracellular acidification. Typically, the pH of the tumour interstitium is 6.2–6.5, while the pH of normal tissue interstitium is neutral (Gerweck 2000; Gerweck et al. 2006). The major type of proton pumps used by cancer cells to maintain their neutral cytosolic pH is the class V ATPase (Sennoune et al. 2004). This ATPase has relatively low activity in nonmalignant cells, while its activity is increased in cancer cells (Izumi et al. 2003; Bowman and Bowman 2005). These observations led to the development of a novel anticancer strategy by inhibiting the proton pumping activity of the ATPase, causing acidification of the cancer cell cytosol that, in turn, results in the demise of the cell. For example, chondropsin compounds (Bowman et al. 2003) and siRNA targeting the ATPase subunit ATP6L (Lu et al. 2005) have been successfully used to kill cancer cells. Other important regulators of cytosolic pH are the Na^+/H^+ antiporter (Slepkov et al. 2007), the H^+/lactate symporter (Cardone et al. 2005) and the Na^+-dependent Cl^-/HCO_3^- exchanger (Lee and Tannock 1998). Similarly as for V-class ATPase, these transporters have been proposed as targets for anticancer drugs (Izumi et al. 2003; Cardone et al. 2005; Harguindey et al. 2005).

The difference in pH gradient across the plasma membrane of cancer cells has been used to design a class of drugs that can be classified as weak acids, with pK_a values of <6, and which are, typically, deprotonated at neutral pH but accept a proton at the pH of the tumour interstitium (Gerweck et al. 2006). A prototypic example of such a drug is the weak acid chlorambucil (Skarsgard et al. 1991). It was reported that the relatively selective uptake and anticancer efficacy of chlorambucil could be enhanced by injection of glucose into mice with experimental tumours, thereby further promoting glycolytic activity of tumour cells, lowering the tumour interstitium pH while not affecting the pH of the tumour cell cytosol (Kozin et al. 2001).

We have observed similar effects for the mitocan α-tocopheryl succinate (α-TOS), a compound with pK_a of 5.6 (Neuzil et al. 2002). The vitamin E analogue is a weak acid, of which ~98% is deprotonated at neutral pH with 10–15-fold higher percentage in the protonated form at the acidic pH of 6.2–6.4 of the tumour interstitium. Since there are no known transporters for compounds like α-TOS, presumably it crosses the plasma membrane to freely diffuse inside the cells and discharge its apoptogenic activity. Accordingly, we found that when the pH of the tissue culture medium was more acidic (pH ~ 6.2), it resulted in about three times greater apoptogenic efficacy of α-TOS against T lymphoma cells compared with media at pH of 7.2. The likely reason is the faster uptake of the compound at lower pH, since we found that about twice as much α-TOS enters at pH 6.2 than when the pH of the medium was 7.4 (Neuzil et al. 2002). Thus, the pH differential across tumour plasmamembranes may be an important paradigm for targeted delivery whereby certain anticancer agents exert selectivity for malignant tissues.

ROS, HIF Activation and Changes in Cytochrome C Oxidase Activity

Oxygen utilization in cells occurs in mitochondria at the endpoint of the respiratory chain, where it acts as a substrate for the cytochrome c oxidase (COX) that constitutes complex IV. Thus, during OXPHOS, the respiratory chain functions to transfer electrons through a series of coupled acceptor systems in a relay that ends in a reaction with oxygen. The electron acceptors contain redox centers (Fe-S complexes, heme groups, FMN, or FAD) in complexes I–IV that also work as proton pumps in order to generate the proton gradient across the MIM (Fig. 2). The potential energy stored in the proton gradient is then used to drive the turbine in the ATP synthase to make ATP. However, the electron transport chain (ETC), as a highly charged system, is not without its containment problems, particularly when coping with overloads or blockages (reviewed in Brookes 2005; Ott et al. 2007). Thus, the single-electron chemistry involved in the ETC predisposes to the generation of reactive oxygen species (ROS) because the redox centers along the respiratory chain can leak electrons to molecular oxygen, producing the superoxide anion (O_2^-).

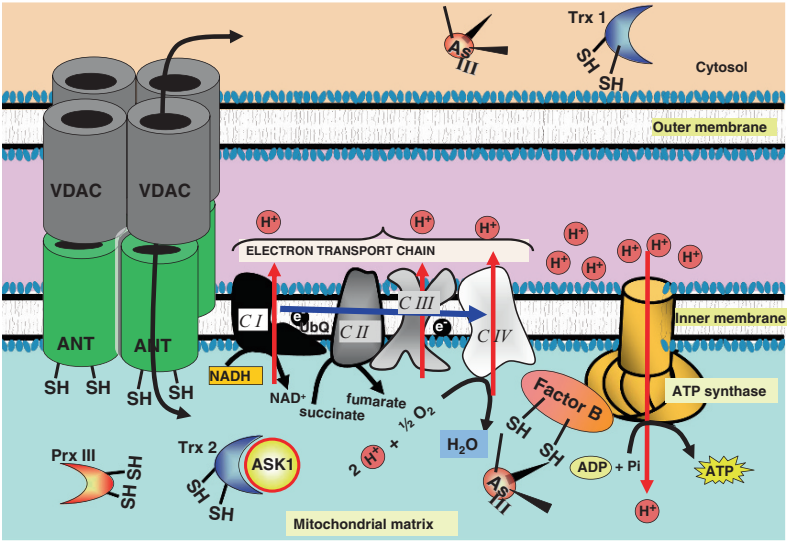

Fig. 2 Many vicinal thiol containing proteins important to REDOX control in the mitochondria are mitocan targets. Arsenite and particularly organic arsenites will disrupt the normal redox systems functioning in the mitochondrial matrix and intermembrane space by targeting vicinal thiols in proteins and enzymes that regulate these systems. Such enzymes include Peroxiredoxin III (Prx III) and Thioredoxin 2 (Trx2). In addition, several key mitochondrial functions are affected, including Factor B regulating the ATP synthetase activity, the adenine nucleotide transporter, ANT, among others. See text for further detail

Studies with specific inhibitors of each of the complexes from I to IV, or with cells containing mutations affecting the activity of these complexes have revealed that either of the complexes, when blocked, is capable of leaking electrons and producing significant levels of superoxide (Turrens 1997; Mclennan and Degli Esposti 2000; Lenaz 2001; Paddenberg et al. 2003; Zuckerbraun 2007). The evidence indicates that the particular source of superoxide production that occurs along the respiratory chain will depend on the type of energy substrate that is predominately being used and the extent of energy/electron flow passing at the time. Enhanced ROS production occurs when the electron carriers in the respiratory chain are kept in the reduced state as a result of either the inhibition of OXPHOS or because of excess energy substrate availability for use by the respiratory complexes. Hence, electrons residing in the unpaired electron of ubisemiquinone bound to the CoQ binding sites of either of complexes I–III are capable of reacting directly with O_2 to produce superoxide anion as a toxic by-product of OXPHOS.

In hyperpolarized cancer cell mitochondria, the electron transport chain stalls because ADP levels drop because of the demands of high glycolysis and conversion to ATP, inhibiting ATP synthase by limiting ADP substrate availability in the mitochondria. Together with reduced passive proton permeability caused by increased sterol production (see earlier text), ATP synthase inhibition greatly restricts the proton flow back to the matrix either across the membrane or via the ATP synthase proton channel. However, the respiratory chain continues to use energy substrates and pump protons across the MIM into the matrix, increasing the transmembrane potential until the $\Delta\Psi_{m,i}$ becomes energetically unfavourable, when further proton flow becomes limited. At the point of the reservoir overload, the chain stalls and the electron carriers become maximally reduced (Wallace 2005). As a result, ubiquinones become extensively converted by absorbing protons to form abundant ubisemiquinone radical anions that then favor alternative reactions with molecular oxygen, producing superoxide, leading to increased oxidative stress and ROS production. Ubisemiquinone anions play an important role in electron transfer to oxygen from complex I (Ohnishi and Salerno 2005), complex II (Xu et al. 1987), and complex III (Yu et al. 1980; Cape et al. 2007). The importance of ubisemiquinones as the source of superoxide production was first recognised by Lehninger in studies of complex III of heart mitochondria (Turrens et al. 1985) and confirmed more recently by Cape et al. (2007).

It follows that control of the redox state of the respiratory chain components will be governed by the transmembrane proton gradient (ΔpH) and the membrane potential $\Delta\Psi_{m,i}$ (Brookes 2005) that reflect the levels of OXPHOS and flux along the electron transport chain. Furthermore, any agent that inhibits OXPHOS when the $\Delta\Psi_{m,i}$ is high will result in increased superoxide production via reaction of ubisemiquinones with molecular oxygen.

Recent evidence has revealed that the COX activity in complex IV of the respiratory chain is regulated as a homeostatic response to enable cells to cope with the demands made by varying oxygen levels. Such changes can cause electron flux problems within the chain components, resulting in aberrant levels of ROS production as described earlier. Intriguingly, in a paradoxical relationship, HIF-1

activation requires ROS production by the electron transport chain (Chandel et al. 2000; reviewed in Bell and Chandel 2007). However, HIF-1 activation induces changes in the regulatory subunit of the complex IV COX, causing a switch in the catalytic subunit to a different isoform. The net effect is an increase in the activity of the oxidase, optimizing the flow down the respiratory chain, ATP production, and O_2 consumption, which helps to prevent ROS production from occurring during hypoxic conditions (Fukuda et al. 2007; Campian et al. 2007). Nevertheless, despite this homeostatic mechanism to avoid excessive ROS production, cancer cells maintain their high $\Delta\Psi_{m,i}$ and low OXPHOS and hence they have a significant ability to generate ROS. This is likely to be further exacerbated by the accumulation of mtDNA mutations and the effects of oncogenes in enhancing HIF expression and glycolysis, while reducing OXPHOS (Wallace 2006).

The dependence of ROS generation on $\Delta\Psi_{m,i}$ and on the reduced state of pyridine nucleotides indicates that under conditions where the ATP synthase is nearly inactive and not bypassed by uncouplers, the high $\Delta\Psi_{m,i}$ and the relatively high NADH:NAD^+ ratio will all contribute to promote increased ROS production (Adam-Vizi 2006).

Importance of ROS in Tumour Cell Development and Progression and the Residual Respiratory Function of Cancer Cells

Previous sections emphasize the importance of ROS for cells to undergo genetic changes leading to transformation and to changes in energy metabolism and mitochondrial function that are associated with malignancy. Superoxide may also play another important role since it has recently been found to directly inhibit the mitochondrial form of aldehyde dehydrogenase (ALDH2), which is the major species involved in metabolizing the carcinogenic acetaldehyde into acetic acid (Dietrich et al. 2007). In support of the significance of the role played by acetaldehyde in cancer, people with a defective allele of the ALDH2 gene who consume alcohol show a much greater predisposition to squamous cell carcinomas of the upper digestive tract (Dietrich et al. 2007).

It appears that a common feature of cancer cells is that they are highly selected and able to survive large transitions in redox state brought about as a result of switching/cycling between intermittent periods of normoxia and ischemia. Mutations resulting in altered mitochondrial function and increased expression of pro-survival proteins also enable the cells to overcome stressful growth conditions/environments, forcing a greater dependency on glycolysis or other sources of metabolic energy. Despite this ability of cancer cells to survive and grow under conditions of low or reduced oxygen levels, they still maintain a residual capacity for aerobic respiration (Warburg 1956).

Whether a requirement of cancer cells to continue maintaining their low residual levels of oxidative respiration is an absolute necessity for their continued survival has not been thoroughly examined. However, in agreement with this notion, cancer cells are very sensitive to mitochondrial respiratory poisons—a situation that should not occur if cancer cells are not dependent upon residual mitochondrial respiration for survival. For example, a study with leukaemia cells (Pelicano et al. 2003) outlined a strategy to hinder mitochondrial electron transport and increase superoxide (O_2^-) radical generation inside these cells as a novel and selective means for enhancing apoptosis induced by anticancer agents. This strategy initially established the proof-of-principle by using rotenone, a specific inhibitor of the NADH oxidoreductase in complex I of the mitochondrial electron transport chain. The resulting partial inhibition of mitochondrial respiration increased electron leakage from the transport chain, leading to an increase in superoxide generation. The net effect was to sensitize the leukemia cells to anticancer agents whose action involves free radical generation. Pelicano et al. (2003) showed that As_2O_3, a clinically useful anti-leukemia agent that inhibits mitochondrial respiratory function, also increased free radical generation and enhanced the activity of the other superoxide-generating agents against cultured leukemia cells and primary leukemia cells isolated from patients.

An additional impact of defects in mitochondrial respiration operates via the cytosolic Akt kinase pathway that promotes cancer cell growth and survival (Pelicano et al. 2006). Disruptions of mitochondrial function, like those already described earlier cause major increases in cellular NADH:NAD^+ ratios. The increase in NADH leads to activation of the Akt pathway via NADH-mediated inactivation of the PTEN phosphatase. Pelicano et al. simulated this situation using respiration-deficient (ρ^0) cells lacking mtDNA and that were largely dependent on glycolysis for their energy needs. These ρ^0 cells produced increased levels of NADH that activated Akt, resulting in drug resistance and survival advantages in hypoxia. Pharmacological inhibitors of mitochondrial respiration or conditions of hypoxia activate Akt in a similar manner. Their studies revealed that conditions where cells lacked mitochondrial respiration (ρ^0 cells) or when chemicals were used to inhibit the electron transport chain (ie pseudohypoxia), or normal cells were exposed to hypoxia, all resulted in significant Akt activation (Pelicano et al. 2006). Additional evidence presented by the same authors showed that the cellular NADH/NADPH ratio increased abnormally when mitochondrial respiration was suppressed, in parallel with a decrease in plasma membrane-associated PTEN. Further, addition of exogenous NADH led to inactivation of PTEN and activation of Akt. The inactivation of PTEN results from redox modulation when NADH competes with NADPH binding to thioredoxin reductase, decreasing the enzyme activity (Larsson 1973). As a result, oxidised PTEN (inactive) is unable to dephosphorylate Akt and inhibit its kinase activity. These results are consistent with previous studies showing inactivation of PTEN by hydrogen peroxide (Lee et al. 2002; Kwon et al. 2004, Connor et al. 2005). Cells lacking functional PTEN did not respond to respiratory inhibition or hypoxia and exhibited no increase in Akt activation, confirming the important role of PTEN in Akt regulation (Pelicano et al. 2006).

NADH produced by glycolysis and the TCA cycle drives OXPHOS via complex I, as does NADPH produced via the pentose phosphate pathway. The proportion of energy substrates, such as glucose, that will be directed down either the glycolytic/TCA or alternatively down the pentose phosphate pathway is regulated by the metabolic state of the cells. We have described earlier how mitochondrial defects in OXPHOS make cancer cells dependent on glycolysis for ATP supply. When OXPHOS is restricted as in cancer cells, the NADH produced from the TCA cycle is no longer used to drive complex I and the respiratory chain. Hence, increased NADH is available for alternative processes such as conversion of pyruvate to lactate. Overall, NADH accumulates while the levels of NADPH relative to NADH decline because of the increased utilization of glucose for glycolysis. Indeed, Pelicano et al. (2006) consistently observed that the NADH/NADPH ratio significantly increased in eight independently derived clones of ρ^0 cells. These authors also showed that the antioxidant *N*-acetyl cysteine (NAC) suppressed Akt activation induced by H_2O_2, but did not decrease rotenone-induced Akt phosphorylation. They proposed that the redox-sensitive PTEN was inactivated when the ratio of NADH/NADPH was significantly increased and that the elevated ratio could not be modulated by NAC when cells were treated with rotenone. In contrast, NAC effectively decreased H_2O_2 and its direct effect on PTEN.

The PI3K–Akt pathway is critical for cell survival (Cantley 2002; Vivanco and Sawyers 2002; Manning and Cantley 2007). Activation of PI3K results in generation of PIP3, which leads to activation of phosphoinositide dependent kinase-1 (PDK-1) and phosphorylation of Akt. In contrast, the lipid phosphatase PTEN removes a phosphate from PIP3, and acts as a negative regulator of Akt. Loss of PTEN leads to Akt activation in cancer cells (Wu et al. 1998). It is likely that oxidation of PTEN suppresses its phosphatase activity, leading to Akt activation. The demonstration that respiration defects lead to activation of the Akt pathway in response to the accumulation of NADH and inactivation of PTEN reveals an important adaptive mechanism enabling cancer cell survival under respiration-compromised conditions. These findings also provide a novel mechanistic insight into the Warburg effect and how metabolic changes in cancer cells reduce sensitivity to therapeutic agents, providing them with a survival advantage.

The Relationship between Thiol Redox Exchange, ROS and Induction of Apoptosis

Studies with a variety of arsenical compounds have implicated protein thiols and thiol reactive systems such as thioredoxins, thioredoxin reductases, glutathione and glutathione reductases in regulating apoptosis and ROS availability in cells. Many of these arsenical compounds react with mono and dithiol groups, particularly the latter when two thiols are in proximity, acting to crosslink the thiols. Consequently, the different arsenical compounds serve as excellent agents for probing and perturbing the thiol-redox systems in cancer cells. In the previous section it was

pointed out how mitochondria and the electron transport chain are the main source of ROS in cells (also reviewed in Andreyev et al. 2005) and may be the key point of thiol-redox exchange affecting cancer cell death. Thioredoxin (TRX), NADPH and thioredoxin reductase (TxR) comprise the thioredoxin system that regulates a wide range of functions in cells, including redox signalling via interactions with other proteins, transcriptional regulation, control of the reduced intracellular redox environment, cell growth, defense against oxidative stress and control of apoptosis (reviewed in Arner and Holmgren 2006). As mentioned previously, the thioredoxin system is very sensitive to arsenic-based drugs and this may be the basis of one of the important mechanisms for their actions in inducing cancer cell death. The TRX system operates as a thiol-disulfide exchange reaction (see Figs. 2, 3). TRX1 and TRX2 are key regulatory isozymes that catalyse the reduction of protein disulfide bonds. They are cofactors of the apoptosis signal-regulating kinase 1 (ASK1) that mediates TNF- and oxidative stress-induced apoptosis via the mitochondrial dependent pathway (Zhang et al. 2004).

In their reduced forms, cytosolic TRX1 and mitochondrial TRX2 each contain vicinal thiol groups in their active site sequence as –C-G-P-C-. TRX1 and TRX2 bind to Cys250 and Cys30, respectively, in the regulatory N-terminal domain of ASK1 to maintain the enzyme in an inhibited state. Activation by TNF results in increased production of ROS, which oxidises the TRX dithiol group to a disulfide. Under these conditions, the thioredoxins no longer bind to ASK1, and loss of TRX2 binding to mitochondrial-located ASK1 leads to apoptosis in a JNK-independent manner. Upon loss of TRX1 binding, cytosolic ASK1 activates as a MAPKKK causing JNK activation, Bid cleavage and translocation of Bax to mitochondria (Zhang et al. 2004). Since it is known that thioredoxins are major targets of arsenic-containing compounds (see earlier text and Hansen et al. 2006), it can be predicted that arsenical-mediated oxidative binding to thioredoxins will have a similar outcome as TNF signalling, leading to the release and activation of ASK1 and induction of apoptosis. It is also likely that the thioredoxins in the same manner will be important sensors of redox changes induced by many anticancer drugs akin to the arsenical compounds that mediate their actions in cancer cells by affecting thiol exchange, redox reactions and ROS production.

Another thioredoxin-associated protein important in thiol mediated redox regulation in mitochondria is thioredoxin peroxidase II (TPX-II, also known as peroxiredoxin III, Prx-III), which is abundantly expressed in the mitochondria of cancer cells and protects the cells from oxidative stress (Nonn et al. 2003; Chang et al. 2004). PrxIII is an important antioxidant that acts in conjunction with TRX2/TXR (Fig. 3) in the mitochondria to remove peroxides such as H_2O_2 to negate the apoptosis-inducing effects of increasing levels of H_2O_2. PrxIII contains three Cys residues, two of them involved as redox-active sites in the formation of a stable inter-subunit disulfide-bonded dimer, which is reduced by thioredoxin to the monomer. PrxIII is a more abundantly expressed arsenic-binding protein in arsenic resistant cells compared with normal cells when measured by phenylarsine oxide affinity chromatography (Chang et al. 2003). Hence, PrxIII is another protein whose function is likely to be inhibited by arsenic-containing compounds promoting apoptosis.

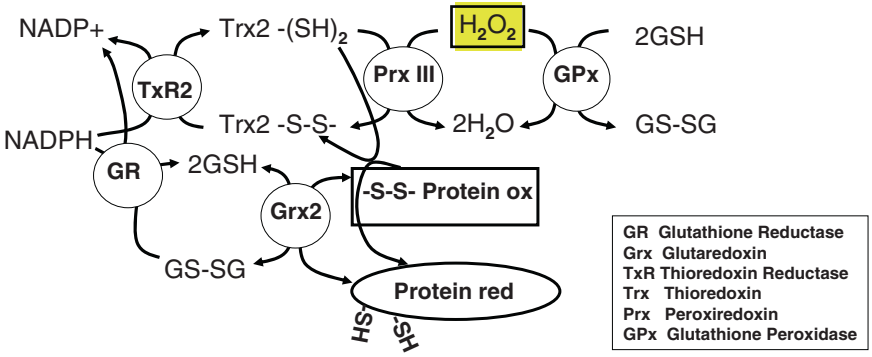

Fig. 3 Mitochondrial redox systems regulating ROS levels via thiol-disulfide exchange/coupling reactions. The mitochondrial form of Thioredoxin (Trx2) is likely to play the major role in reducing disulfides formed by vicinal thiols in both the mitochondrial Peroxiredoxin III (Prx III) and other proteins. Prx III is one of the main ways by which cancer cells can reduce the build up of H_2O_2 as a result of overload from electron transport flow. The glutathione redox system comprising GSH/GSSG, glutathione reductase, glutaredoxin and glutathione peroxidase, although present in the mitochondria, are more likely to only become of major importance during the more extreme conditions of oxidative stress. Both of these systems are targets for inhibition by arsenic-containing compounds and other mitocans. See text for further detail

It is apparent that dithiol-containing redox proteins, particularly those present in mitochondria, act as control sensors during responses to changes in the cellular redox state. Many redox-active proteins contain one or more vicinal pairs of reactive thiol groups (Fomenko and Gladyshev 2003; Fomenko et al. 2007) and many of these redox proteins will be capable of binding arsenic-containing compounds in a similar manner to those of the thioredoxin system. The most important dithiol-containing redox proteins are located in mitochondria, providing high sensitivity to arsenic-containing compounds whose actions culminate in triggering apoptotic pathways in cancer cells via the induction of reactive oxygen species (Fig. 4). Two members of the glutaredoxin (Grx) family, including Grx2 located primarily in the mitochondria, catalyse GSH-dependent TRX-disulfide redox and protein thiol-disulfide redox reactions, in particular the reversible glutathionylation of protein sulfhydryl groups (Berndt et al. 2007). Human Grx1 and Grx2 contain C-P-Y-C and C-S-Y-C active sites as well as three and two additional structural Cys residues, respectively. Therefore, they are also likely to react with arsenic-containing compounds.

Another mitochondrial protein targeted by arsenic-containing compounds with a vicinal dithiol group is the regulatory protein "Factor B". Addition of recombinant Factor B back to bovine submitochondrial particles depleted of this protein restored the energy coupling activity. Thus, reverse electron transfer from succinate to complex I enabling NAD^+ reduction, electron transport chain function and OXPHOS/^{32}Pi-ATP exchange of the ATP synthetase complex were reactivated (Belogrudov and Hatefi 2002) and exchange activity of complex V was increased (Belogrudov 2002). The F0-F1 ATPase activity requires Factor B coupled to it for full activity (see Fig. 2). However, Factor B contains six thiols, and Cys 92 and Cys

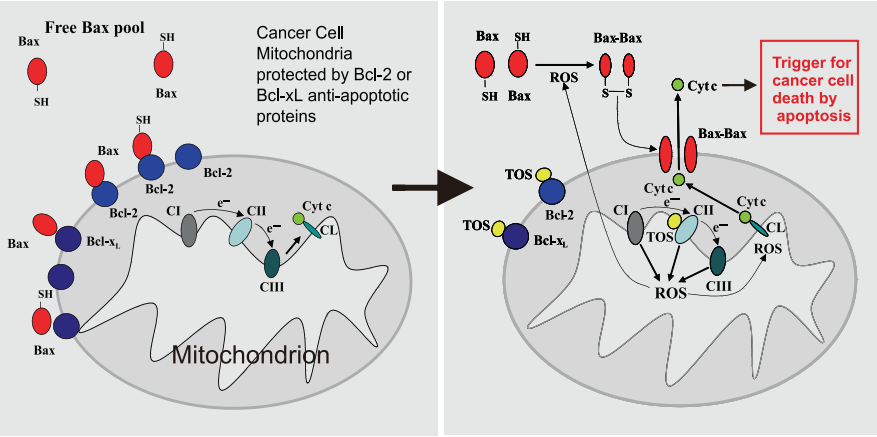

Fig. 4 Universal model for the molecular mechanism of cancer cell apoptosis initiated by mitocans via ROS production as exemplified by the drug, α-tocopheryl succinate (α-TOS). According to this model, there are at least two roles for mitocan mediated cell death: the major role involves the drug inhibiting oxidative respiration at the level of electron transport complexes, and an ancilliary role, which involves binding and blocking Bcl-2 and Bcl-xL function to allow Bax to form mitochondrial membrane channels. Thus, for example, α-TOS, as shown in the right hand panel, upon inhibiting the activity of complex II, impairs electron transfer flowing along the redox chain. This leads to generation of ROS, such as the freely diffusible superoxide anion radicals. ROS then contribute to the ensuing events leading to cell death. The cysteine residues on Bax monomers are oxidised to form disulfide bridges, whereby the protein dimerises. This changes the conformation of Bax, so that the mitochondrial docking motif is exposed and the dimers merge in the mitochondrial outer membrane (MOM), forming multimeric channels. ROS also oxidise cardiolipin (CL) within the mitochondrial inner membrane (MIM). This leads to dissociation of cytochrome c (Cyt c) from the intermembrane face of CL. Free Cyt c then escapes via the Bax channels in the MOM to the cytoplasm. α-TOS, occupying the BH3 domains of Bcl-2 and Bcl-xL, prevents Bax from forming inactive oligomers with Bcl-2 and Bcl-xL, thereby increasing the pool of available Bax dimers that form MOM channels promoting induction of apoptosis

94 in the bovine protein were shown to bind phenylarsine oxide (Belogrudov 2006), and phenylarsine oxide or arsenite inhibited Factor B-coupling activity (Joshi and Hughes 1981). From all of these studies, it is becoming clear that redox changes to vicinal thiols affect regulation of the mitochondrial function and that these thiols also provide major targets for inhibition by thiol reactive mitocans, including arsenic-containing compounds.

The Adenine Nucleotide Transporter (ANT): Critical Thiol Groups as Targets of Arsenic-Containing Compounds in the Mitochondria

A channel formed by the association of two proteins, VDAC in the MOM and ANT in the MIM (Fig. 2), is a complex involved in the induction of apoptosis activated via the mitochondrial pathway. The two components of this complex form part of

the mitochondrial permeability transition pore (MPTP), a channel mediating release of molecules from mitochondria that activate apoptosis. The rapidly increasing permeability of the MIM (mitochondrial permeability transition) proceeds to apoptosis mediated by the MPTP.

Arsenite induces apoptosis by a direct effect on the MPTP (Larochette et al. 1999; Verrier et al. 2004), and VDAC has been shown to play a role in opening of the permeability pore and cytochrome c (Cyt c) release induced by arsenic trioxide, which also causes VDAC to homodimerise (Zheng et al. 2004). In addition, the thiol-reactive compound 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) has been shown to block the VDAC channel (Shafir et al. 1998) and inhibit ROS-mediated Cyt c release (Madesh and Hajnoczky 2001). This data suggests that VDAC may contain critical Cys residues that can form intermolecular cross-links following reaction with arsenical compounds. Analysis of ion channel activity of purified ANT-containing lipid bilayers also revealed that arsenic trioxide altered the ANT channel electrophysiological properties (Belzacq et al. 2001). Interestingly, glutathione depletion leading to increased ROS may play an important role in the action of arsenic trioxide (Verrier et al. 2004). Although in both normal and cancer cells, glutathione S transferase (GST) is found to interact with ANT, during the induction phase of apoptosis, GST dissociates from ANT, suggesting that GST/GSH may act as a repressor of MPTP and ANT pore opening (Verrier et al. 2004; Zhou et al. 2005). This possibility is supported by the observation that increasing the expression of GSTP1 in Jurkat and Raji cells renders them more resistant to arsenic trioxide-induced apoptosis at clinically relevant levels. GSTP1 expression in these cells was also accompanied by decreased production of H_2O_2 (Zhou et al. 2005).

Facing inside the mitochondrial matrix, ANT has three exposed loops containing a conserved repeat structure with one Cys residue in each loop. These residues are important to the process of ANT dimerisation, but it is not clear how this operates and whether the Cys residues form intermolecular disulfide bonds (Dyall et al. 2003). Nevertheless, copper-*o*-phenanthroline is able to dimerise ANT by intermolecular cross-linking of Cys 56 (in the first matrix loop) (McStay et al. 2002). In addition, phenylarsine oxide, eosin 5-maleimide and diamide form intramolecular cross-links between Cys 160 and Cys 257 on the other two matrix loops, restricting ANT in the open conformation and promoting mitochondrial permeability transition (McStay et al. 2002). Arsenic trioxide is much weaker than phenylarsine oxide at binding to the ANT Cys residues (Costantini et al. 1996; Costantini et al. 2000) and this may explain the greater sensitivity exhibited by acute promyelocytic leukemia cells to phenylarsine oxide than to As_2O_3 (Sahara et al. 2004).

Single thiol-interacting compounds, such as *N*-ethylmaleimide (NEM), can inhibit the mitochondrial permeability transition. This could occur either as a result of direct interaction with the key Cys residues on the matrix loops of ANT, or indirectly via reaction with GSH preventing oxidation of GSH that would catalyse disulfide bridging between the adjacent thiol groups in the ANT loops (Mcstay et al. 2002). NEM or monobromobimane preferentially react with GSH, leading to its modification in mitochondria and thereby preventing GSH from oxidation. As a result, NEM inhibits mitochondrial permeability transition activation by the thiol

reactive compounds, diamide or *t*-butylhydroperoxide, implying a role for GSSG in the action of these agents on the permeability transition (Costantini et al. 1996; Costantini et al. 2000). Arsenites, albeit at higher concentrations given their lower affinity for glutathione, could exert a similar action. Thus, high levels of arsenites might modify and inhibit glutathione redox control so that glutathione-based enzymes are unable to function, as well as directly mediating disulfide cross-linking of ANT and increasing cellular ROS production, MPTP and apoptosis. However, given their low reactivity with glutathione systems, this scenario appears to be less likely than the indirect action via mitochondria leading to increased ROS production, which then reduces cellular GSH levels.

The Importance of ROS Production by Mitocans in Triggering Apoptosis: a General Model

Since many of the mitocans rely on their ability to act as pro-oxidants in mitochondria of malignant cells, it follows that the production of ROS by these organelles is very likely to be an important trigger leading to the activation of cancer cell death by the apoptotic signalling pathway, a process mediated by the mitochondria. The general mechanism whereby ROS activate the mitochondrial apoptotic pathway has not yet been definitively resolved. However, we believe that a fundamental universal process whereby mitocans induce cancer cell death is emerging from the available evidence and we recently proposed a plausible model to explain the process (Neuzil et al. 2006). Although superoxide is the immediate product from the respiratory chain leakage of electrons, it is converted to hydrogen peroxide (H_2O_2) by the superoxide dismutase within mitochondria. In particular, the superoxide released from ubisemiquinone on the MIM face of the respiratory chain (Han et al. 2001, 2002) reacts with Cu,ZnSOD that is located there (Slot et al. 1986). The interaction with superoxide activates Cu,ZnSOD to produce H_2O_2 (Inarrea et al. 2005), which is then released across the MOM into the cytosol (Han et al. 2003). The Cu,ZnSOD activity can be inhibited by TxR located in the MIM (Inarrea et al. 2007). The H_2O_2 produced then affects many processes within the mitochondria as a major signal for the onset of apoptosis.

Numerous reports have proposed various roles for ROS in apoptosis although ROS probably has two major roles in mitochondrial translocation of Bax and cytosolic mobilisation of Cyt c. In a healthy cell, monomeric Bax is located predominantly in the cytoplasm, with the C-terminal mitochondrial docking motif hidden owing to its conformation. Upon initiation of apoptosis, Bax conformation changes exposing the docking motif and provoking dimerisation of Bax, which then moves to the MOM, where Bax aggregates to form pores (Degli-Esposti and Dive 2003). Several domains within the Bax molecule are critical for Bax oligomerisation and movement to the MOM, where Bax forms trans-membrane (TM) channels. These include the Bcl-2 homology-3 (BH3) domain close to the N-terminus essential for dimerisation of Bax (Wang et al. 1998; Cartron et al. 2005), and the

C-terminal TM or mitochondrial docking domain important for mitochondrial recognition of Bax homodimers (Wolter et al. 1997). It also appears that the Bax BH3 and TM domains interact with each other during apoptosis signalling (Nechushtan et al. 1999). Both dimerisation and exposure of the TM domain occur before translocation of Bax to mitochondria (Antonsson et al. 2001), consistent with these events occurring early during apoptogenic signalling.

Other studies have reported that Bax dimers formed in the cytoplasm move to the MOM, where they associate into tetramers and octamers that allow larger molecules, such as Cyt c, to pass through, and that these "megachannels" do not include VDAC (Antonsson et al. 2001; Mikhailov et al. 2001). It has been shown that Bax dimerisation and mitochondrial translocation proceeds prior to mitochondrial events (Smaili et al. 2001; Pagliari et al. 2005) and that the chaperone HSC70, that maintains proteins in an unfolded state, prevents Bax translocating to the cytosolic face of the MOM (Stankiewicz et al. 2005). Recent evidence suggests that ROS production causes dimerisation of Bax in the cytosol by generating disulfide bridges between critical cysteine residues (D'Alessio et al. 2005). The ROS originates from the initial mitochondrial events in the intrinsic apoptogenic signalling, both in whole cells and in mitochondria-free cytosolic fractions. Computer simulations have shown that these disulphide bridges change the conformation of Bax, so that it translocates to the MOM (D'Alessio et al. 2005). Bax contains two Cys residues that are exposed, accessible to oxidants (Suzuki et al. 2000). Molecular modelling has suggested that the most favourable disulfide bridges in Bax dimers would be formed by linking Cys162-Cys162 or Cys162-Cys62. The latter combination is the more likely since Cys62 is within the BH3 domain of Bax that is essential for Bax dimerisation and conformational change, as well as for the interaction of Bax with the Bcl-2 and Bcl-x_L proteins (Zha et al. 1996; Yethon et al. 2003). However, further experimental validation of this proposal is still required. Notwithstanding, this is the first and very important indication of the molecular mechanism of Bax dimerisation and translocation in a system where apoptosis is induced by oxidative stress.

Another central player in mitochondrial-dependent apoptosis, leading to caspase activation, is Cyt c (Zhivotovsky et al. 1998). This mitochondrial intermembrane protein is anchored to the MIM owing to its affinity for the mitochondria-specific phospholipid cardiolipin (CL), and the binding is disrupted upon oxidation of CL by ROS derived from the OXPHOS complexes (Petrosillo et al. 2001; Ott et al. 2002). Oxidation of CL and subsequent release of Cyt c are both prevented by endogenous SOD (Petrosillo et al. 2001) or exogenous cell-permeable antioxidants (Zhao et al. 2004). Furthermore, Cyt c alone appears to have an oxidase activity that results in modification of CL and release of the protein from CL. Using oxidative lipidomics, Kagan et al. (2005) showed that CL was the only phospholipid in mitochondria oxidised in the early stages of apoptosis initiated by staurosporine or actinomycin D, and that it was triggered by mitochondria-dependent generation of ROS. Finally, Perier et al. (2005) have presented evidence that cells deficient in complex I generate ROS, leading to CL oxidation and subsequent Cyt c detachment from this phospholipid, followed by cytoplasmic relocalisation of Cyt c via Bax channels (Zha et al.

1996). Collectively, these results link mitochondrial ROS production, ROS-dependent Bax dimerisation and formation of channels in the MOM and release of Cyt c from its binding to the intramembrane face of the MIM into the cytoplasm.

Mitochondrial proteins Bcl-2 and Bcl-x_L exert their anti-apoptotic activity by diverting Bax and preventing it from forming MOM channels. These anti-apoptotic Bcl-2 family members contain BH3 binding domains in the form of a hydrophobic groove exposed on the cytosolic face of the MOM, and they both bind Bax via the Bax BH3 domain (Chang et al. 1997; Perier et al. 2005). Since heterodimers between Bax and Bcl-2/Bcl-x_L are favoured over hetero-tetramers (Zhang et al. 2004), it is possible that Bcl-2 and Bcl-x_L capture Bax monomers before they dimerise become "activated", thereby preventing Bax from forming channels in the MOM (Tan et al. 2006). A recent publication reported a novel function of one of the mitocans, α-tocopherol succinate (α-TOS), in that it associates with the same hydrophobic groove on Bcl-2 or Bcl-x_L (Shiau et al. 2006). Thus, α-TOS binding prevented capture of the Bax-homologue Bak by the two anti-apoptotic proteins, thereby increasing the pool of Bak required for the formation of channels in the MOM. Since Bak also binds to the BH3 domains of Bcl-2/Bcl-x_L, it is possible that α-TOS will, in an analogous manner, release Bax to form MOM channels. Moreover, α-TOS induced ROS production would facilitate the ROS-dependent dimerisation of Bax, otherwise monomeric Bax would be captured via the hydrophobic pocket on the cytosolic side of unoccupied Bcl-2 or Bcl-x_L (Zhang et al. 2004; Tan et al. 2006).

Types of Mitocans and their Targets

We have catalogued the mitocans into seven different classes depending on their particular target(s) associated with mitochondrial function and each class of mitocans is covered separately, exploring their mechanism of action and applications to date. Numerous drugs have been described that target mitochondria and induce apoptosis of cancer cells. However, only those whose site of action has been clearly identified are discussed in the present review. The different classes of mitocans encompass a growing number of drugs listed in Table 1. Classification relates to the different metabolic reactions associated with the mitochondrial organelle whose function is central to cell survival. Grouping the mitocans into different classes helps to rationalize the variety of mitocans and is based on the order and level from outside to inside mitochondria and their site of drug action. If one of these reactions is inhibited inside the cancer cell, then continued survival becomes compromised. The basis for the specificity of these drugs for cancer cells is examined in the following sections where examples from each class are discussed.

Table 1 Compilation of the different classes of mitocans based on their particular mitochondrial target sites that exist in, or are associated with, cancer cell mitochondria and their distinctive properties

Mitocan class	Site of action	Drug names	References
I: Hexokinase inhibitors	Hexokinase HK-1 or HK-II	3-Bromopyruvate 2-Deoxyglucaose	Mathupala et al. 2006
II: BH3 Mimetics	Bcl-2, Bcl-xL	Gossypol, EGCG, antimycin, α-tocopheryl succinate, HA14–1	O'Neill et al. 2004; van Delft et al. 2006; Shiau et al. 2006; Antignani and Youle 2006; Robey and Hay 2006
III: Thiol redox inhibitors	VDAC, ANT, GST, TRX	Isothiocyanates, PEITCs, phenylarseneoxides, arsenites, Cu-phenanthrolines	Shafir et al. 1998; Costantini et al. 2000; Madesh et al. 2001; McStay et al. 2002; Sahara et al. 2004; Hirano et al. 2005; Don et al. 2003; Trachootham et al. 2006; Xiao et al. 2006
IV: VDAC/ANT targeting (not via –SH)	VDAC, ANT	Lonidamine, bisphosphonates, MT21, steroid analogues like CD437, ATRA	Belzacq et al. 2001; Monkkonen et al. 2006; Garattini et al. 2004
V: Electron transport chain (ETC)-targeting Drugs	Complex I, II, III or IV	α-Tocopheryl succinate, 4-OH retinamide. Tamoxifen, resveratrol, dicumarol	Hail and Lotan 2006; Kallio et al. 2005; Tinhofer et al. 2001; Gosslau et al. 2005; Sareen et al. 2006; Zunino and Storms 2006; Ko et al. 2004; Neuzil et al. 2007
VI: Lipophilic cations targeting the mitochondrial inner membrane	MIM	Rhodamine 123, MKT-077, dequalinium, F16, (KLAKKLAK) 2 peptide	Bernal et al. 1982; 1983; Davis et al. 1985; Lampidis et al. 1985; Smith et al. 2003; Fantin et al. 2002; Ellerby et al. 1999; Fantin et al. 2005
VII: Drugs targeting other sites	Unknown	Betulinic acid, phenoxodiol, sesquiterpene lactones (parthenolides)	Maaser et al. 2005; James et al. 2006; Kessler et al. 2007; Rzeski et al. 2006; Nakagawa et al. 2005; Guzman et al. 2005

Class I: Hexokinase Inhibitors

3-bromopyruvate (3BP) and 2-deoxyglucose (2DG) belong to a class of mitocans inhibiting hexokinases that catalyse the conversion of glucose to glucose-6-phosphate accompanied by hydrolysis of ATP. The hexokinases are predominantly found in cancer cells associated with the outer mitochondrial surface (Mathupala

et al. 2006). There, hexokinases I and II help protect the mitochondrial transmembrane channel formed between the MOM porin, VDAC1 and the MIM ANT (Ralph et al. 2006; Vyssokikh et al. 2004). Hexokinase association with VDAC prevents pro-apoptotic molecules like Bax from binding to the outer face of VDAC and activating the MOM pore and apoptotic signalling pathway. 3BP and 2DG are classed as mitocans because the inhibition they cause reduces the stability of the HK-VDAC association, thereby increasing the propensity for pro-apoptotic molecules like Bax to bind to VDAC and facilitate formation of the MOM pore (Pastorino et al. 2002).

Inactivation of endogenous cyclophilin D with small interfering RNAs or a cyclophilin inhibitor was found to release HK-II from mitochondria and enhance Bax-mediated apoptosis (Machida et al. 2006). The anti-apoptotic effects of cyclophilin D were negated by the detachment of HK-II from mitochondria, demonstrating that mitochondrial binding of hexokinase II is essential to the suppression of apoptosis by cyclophilin D. Furthermore, cyclophilin D dysfunction appears to abrogate HK-II-mediated suppression of apoptosis, indicating that cyclophilin D is required for the anti-apoptotic activity of HK-II (Machida et al. 2006).

Not only does 3BP inhibit hexokinases (Mathupala et al. 2006), but it also inhibits the activity of many other glycolytic pathway enzymes and the TCA cycle/electron transport component, succinate dehydrogenase (SDH) (Sanborn et al. 1971), such that it is also included in class V of mitocans (see below).

Class II: BH3 Mimetics

This group of mitocans is very diverse, including the polyphenolic drugs like gossypol, the green tea constituent epigalocatechingallate (EGCG) and peptides based on the BH3 domain sequence (O'Neill et al. 2004; van Delft et al. 2006). BH3 mimetics act by targeting the BH3 domain of Bcl-2 and Bcl-x_L. This inhibits Bcl-2 and Bcl-x_L, preventing them from binding to Bax and Bak and blocking these pro-apoptotic proteins from forming pores in the MOM. Consequently, in the presence of excess BH3 mimetic, when Bax and Bak are activated by apoptotic signals, they become free to form the MOM pore and induce apoptosis (Antignani and Youle 2006; Robey and Hay 2006). Although Bax and Bak have been shown to form pores either alone, or in complex with VDAC, the precise mechanisms for this pore forming process are not yet well defined (Shoshan-Barmatz et al. 2006).

An intriguing BH3 mimetic is α-TOS, which has been shown to disrupt interaction of Bak with Bcl-2 and Bcl-xL (Shiau et al. 2006). Since this drug also induces apoptosis by disrupting the electron redox chain by means of displacing CoQ in complex II causing generation of ROS (Neuzil et al. 2006, Dong et al. 2007, 2008) α-TOS belongs to both mitocan class II and V.

Class III and IV: Thiol Redox Inhibitors/VDAC- and ANT-Targeting Drugs

The class III mitocans act by modifying thiol redox states of key molecules that regulate the function of the mitochondrial permeability transition and the mitochondrial transmembrane channel proteins, including VDAC and ANT. Both of these proteins, VDAC and ANT, contain two or more Cys residues that provide reactive thiol groups whose modification affects their function and ANT dimerises to form the nucleotide transporter channel (Dahout-Gonzalez et al. 2006). It has been established that the redox state of thiol reactive groups is important for activation of the mitochondrial permeability transition (Costantini et al. 2000; McStay et al. 2002). Consequently, thiol cross-linkers such as DIDS, diamide and phenylarseneoxide (PAO) affect VDAC and ANT channel function and activation of mitochondrial permeability transition (Shafir et al. 1998; Costantini et al. 2000; Madesh et al. 2001; McStay et al. 2002). Several other compounds also target thiol groups of either VDAC or ANT. For example, copper-*o*-phenanthroline has been proposed to dimerise ANT by intermolecular cross-linking of Cys56 (McStay et al. 2002). It was proposed that PAO, eosin 5-maleimide or diamide form intramolecular cross-links between Cys160 and Cys257, restricting ANT in a state known as the C-confirmation, promoting mitochondrial permeability transition (McStay et al. 2002). Arsenite is much weaker than PAO in modifying the ANT Cys residues (Sahara et al. 2004). However, PAO is a highly toxic compound in vivo, is nonselective for cancer cells (Hirano et al. 2005). It is also a strong inhibitor of tyrosine phosphatases (Zhang et al. 1992).

The glutathionyl peptide trivalent arsenical compound 4-(*N*-(*S*-glutathionylacetyl)amino) phenylarsenoxide (GSAO) inactivates ANT-mediated ATP/ADP transport and triggers influx of Ca^{2+} upon cross-linking Cys160 and 257 of ANT. This leads to increased cellular ROS, ATP depletion, mitochondrial depolarisation and apoptosis in angiogenic endothelial cells and inhibited tumour growth in mice with no apparent side effects (Don et al. 2003). Interestingly, the para form of GSAO revealed no apparent toxicity in animals undergoing treatment and inhibited tumour growth, leading to phase I clinical trials as an anticancer agent (Don et al. 2003). However, the ortho form was toxic and this was proposed to result from increased accumulation of the drug in normal cells, possibly due to loss of MDR efflux (Dilda et al. 2005).

Single thiol interacting compounds such as *N*-ethylmaleimide (NEM) can inhibit the mitochondrial permeability transition and this could be either the result of direct interaction with key Cys residues on ANT or indirectly via reaction with GSH, preventing it from being oxidized and catalysing disulfide bridging between adjacent thiol groups in ANT (McStay et al. 2002). NEM or monobromobimane preferentially react with GSH, leading to its modification in mitochondria and thereby prevents GSH from being oxidised. As a result, NEM inhibits mitochondrial permeability transition activation by the thiol reactive compounds, diamide or *t*-butylhydroperoxide, implying a role for GSSG in the action of these agents on the

permeability transition induced by disulfide cross-linking (Costantini et al. 1996). Arsenites may have a similar action, modifying and inhibiting the role of glutathione in redox control, such that glutathione-based enzymes are unable to function, leading to increases in cellular ROS production. GST interacts with ANT in both normal and cancer cells, and becomes dissociated from ANT during apoptosis induction, suggesting that GST/GSH may repress the permeability transition and ANT pore opening (Verrier et al. 2004). This is supported by the observation that increasing the expression of GST in cancer cells renders them more resistant to arsenite induced apoptosis, reducing the levels of ROS (Zhou et al. 2005).

Another group of compounds in the third class of mitocans are the isothiocyanates, particularly the dietary phenyl ethyl isothiocyanates (PEITCs) (Trachootham et al. 2006; Xiao et al. 2006). Again, these compounds are thiol modifiers, forming adducts with thiol groups in important redox regulators. PEITCs effectively inhibit the glutathione antioxidant system, producing excessive ROS accumulation in transformed cells. The excessive ROS output promotes oxidative mitochondrial damage, inactivation of redox-sensitive molecules and massive cell death. In animal cancer models, PEITC was shown to exhibit therapeutic activity and to prolong animal survival.

Several different mitocans in the fourth class, like those in the third class, also affect the function of the ANT ion channels and transporters of nucleotides, but in this case, by directly binding and modifying the protein subunit structure. Oligomeric structures comprising VDAC subunits in the MOM and ANT subunits in the MIM exist in a coupled state forming a channel spanning across the two mitochondrial membranes (Zalk et al. 2005). Drugs in the fourth class of mitocans, that bind ANT include lonidamine, an indazole carboxylate (Belzacq et al. 2001), bisphosphonates that form cytotoxic ATP analogue-type metabolites (Monkkonen et al. 2006), as well as retinoid-like structures (e.g. CD 437) and all-trans retinoic acid (Garattini et al. 2004). Reports of VDAC-binding modifiers acting as mitocans could not be found.

Cations such as ruthenium red and La^{3+} binding to Ca^{2+} sites on VDAC block channel activity and appear to prevent the activation of the MOM pore (Gincel et al. 2001). This may be because Ca^{2+} influx via VDAC into mitochondria is required for the induction of apoptosis and hence, drugs that block Ca^{2+} entry would help prevent apoptosis.

Class V: Electron Transport Chain-Targeting Drugs

The fifth class of mitocans comprises a large number of different drugs that target the components of the electron transport chain, leading to ROS production and activation of apoptosis in cancer cells. These drugs include α-TOS, the main interest of the authors, as well as related drugs such as *N*-(4hydroxyphenyl)retinamide (4HPR, fenretinide). The pro-oxidant mechanisms of 4HPR activity have not

been clearly identified, although it is likely to act in the micromolar range as an inhibitor of at least one of the complexes along the electron transport chain, requiring mitochondrial respiration for its apoptotic activity (reviewed in Hail and Lotan 2006). Tamoxifen was shown to induce apoptosis in MCF-7 breast cancer cells at low levels by affecting the mitochondrial function and increasing ROS accumulation (Kallio et al. 2005). The target of tamoxifen action was later identified as the flavin mononucleotide site of complex I leading to H_2O_2 production.

The vitamin E analogue, α-tocopheryl succinate (α-TOS), selectively induces killing of cancer cells by a process involving the mitochondria and accumulation of reactive oxygen species. The molecular target of α-TOS was not known until recently when we showed that α-TOS works by inhibiting the succinate dehydrogenase activity of complex II in the mitochondrial electron transport chain (Dong et al. 2008). In particular, the drug interacts with the coenzyme Q/ubiquinone (CoQ/UbQ) binding sites on complex II, based on biochemical analyses and computer based molecular modelling (Neuzil et al. 2007). In addition, by genetically modifying cancer cells to knock out their mitochondrial complex II, they became resistant to killing by α-TOS and failed to accumulate ROS. By reconstituting a functional complex II to restore its activity in the defective cancer cells, they again became susceptible to α-TOS killing. Similar resistance to the drug was observed in cancer cells where gene expression of the SDHC subunit of complex II was previously reduced using short interfering RNA molecules (siRNA). We propose that α-TOS works to kill cancer cells by displacing UbQ from binding to complex II in cancer cell mitochondria. As a result, electrons generated by the action of the succinate dehydrogenase/complex II recombine with molecular oxygen to produce high ROS levels. Our data, as well as pre-clinical studies with breast cancer highlight α-TOS targeting of complex II as a potent and selective cancer therapy, with additional lethality owing to its anti-angiogenic effects as well (Dong et al. 2007).

Resveratrol acts in the micromolar range at several different sites from complex I to III along the electron transport chain, probably competing with ubiquinone and can also inhibit the F1 ATPase at these levels (Zini et al. 1999; Gledhill and Walker 2005). Although resveratrol is considered an antioxidant, it can also act as a pro-oxidant by enhancing ROS production in cells, inducing apoptosis via the mitochondrial pathway (Tinhofer et al. 2001). A methoxy derivative of resveratrol showed even more potent activity in inducing apoptosis in transformed cells (Gosslau et al. 2005). Two additional reports with different cancer cell types further support the ability of resveratrol to act as a mitocan (Sareen et al. 2006; Zunino and Storms 2006).

3BP, already mentioned as a class I mitocan, also belongs to this class. It causes a rapid decrease in cellular production of ATP, a result of its inhibition of SDH (Ko et al. 2004). A related drug targeting SDH is 3-nitropropionic acid and this inhibition is associated with a large increase in superoxide as a by-product of the electron transport chain inhibition (Bacsi et al. 2006).

Class VI: Lipophilic Cations Targeting the Mitochondrial Inner Membrane

The sixth class of mitocans includes molecules that are delocalized lipophilic cations, which accumulate at much greater concentrations in the mitochondrial matrix than in the cytoplasm of cells (Smith et al. 2003). These agents are selectively accumulated in the mitochondrial matrix of cancer cells because of their greater transmembrane potentials across the plasma membrane as well as their more polarized mitochondria with a much greater than $\Delta\Psi_{m,i}$ that in nonmalignant cells (Davis et al. 1985; Lampidis et al. 1985; Ralph et al. 2006). The target for the lipophilic cation-based mitocans may be one of the inhibitory binding sites on ATPase (Gledhill and Walker 2005). One of the earliest members of this class of compounds to be identified for its anticancer activity was rhodamine-123 (Bernal et al. 1982; 1983). It recently entered phase I clinical trials for prostate cancer and revealed minimal side effects and safe administration at monthly intervals without detectable drug accumulation in the serum of patients (Jones et al. 2005). It is likely that the related compound, Rose Bengal, works in a similar fashion to rhodamine 123, and Rose Bengal is likewise currently in clinical trials as a therapy for metastatic melanoma and recurrent breast cancer, causing complete remissions in some patients (Provectus PV-10-MM-01, www.ClinicalTrials.gov).

The drug F16 is a mechanistically more characterized example of this mitocan class and was shown to increase ROS production, depolarize mitochondria as a weak protonophore and collapse $\Delta\Psi_{m,i}$ leading to mitochondrial permeability transition and selective apoptosis of cancer cells when applied in the micromolar range (Fantin et al. 2002). F16 was also reported in this study to inhibit the growth of mammary tumours in mice. MKT-077, a rhodocyanine dye analogue is another example of this type of mitocan that entered phase I clinical trials, although these were terminated owing to renal toxicity (Britten et al. 2000).

This raises the issue of toxicity with many of the class VI mitocans. A cautionary example of the potential for toxicity that must be carefully evaluated is the production of Parkinson's-like effects by the drug MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) caused by the selective destruction of the nigrostriatal dopaminergic neurons. This toxicity was due to the selective uptake by the dopamine transporters on these cells as well as metabolites formed by the action of the enzyme, monoamine oxidase B that is highly expressed in the dopaminergic neurons. As a result of these two unique properties of dopaminergic neurons, the mitochondriotoxic drug MPP^+ (1-methyl-4-phenylpyridinium) is produced, which acts as an inhibitor of mitochondrial respiration by blocking the NADH-ubiquinone oxidoreductase site of complex I. It is likely that MPP^+ is also a protonophore (Davey et al. 1992; Albores et al. 1990) that collapses the $\Delta\Psi_{m,i}$ leading to cell destruction. This, together with the nonselective cell toxicity associated with another lipophilic cation and known mitochondrial poison, dequalinium chloride (Gamboa-Vujicic et al. 1993), raises the importance of identifying class 6 mitocans that are cancer cell-

specific in terms of their uptake and cellular toxicity, as recently described in a predictive model based on their structures by Trapp and Horobin (2005).

The amphipathic and positively charged α-helical pro-apoptotic peptide $(KLAKLAK)_2$ has also been included in this class of mitocans as a delocalised lipophilic cation. However, the peptide must first be coupled to a targeted delivery system for surface receptor binding and uptake into cancer cells, before it is able to function as a mitocan (Ellerby et al. 1999; Fantin et al. 2005). As with the other members of this class of mitocans, the peptide has been shown to dissipate $\Delta\Psi_{m,i}$ leading to apoptosis, and it efficiently reduced tumour burdens in animal models (Fantin et al. 2005).

Class VII: Drugs Targeting Other Sites

The last class of mitocans includes those whose target site and mechanism of action on cancer cell mitochondria is not well characterized or at present is unclear. It includes drugs that bind to the peripheral benzodiazepine receptor (PBR) such as PK-11195 and Ro5–4864 (Maaser et al. 2005; James et al. 2006). PBR is also known as the MOM translocator protein (Papadopoulos et al. 2006), an 18 kDa MOM-localized protein involved in transport of cholesterol into mitochondria as the rate-determining, hormone-sensitive step in steroid biosynthesis (Liu et al. 2006). It is also important for porphyrin transport and heme synthesis, apoptosis, cell proliferation, anion transport, regulation of mitochondrial functions and immunomodulation. This protein is often overexpressed in cancer cells (Pretner et al. 2006; Papadopoulos et al. 2006) and is a potential target for anticancer drugs that bind to it, but this area of research has not been well defined.

The other drugs included in this class of mitocans are the natural product derived pentacyclic triterpenoids such as betulinic acid and related structures. Betulinic acid and its derivatives have been shown to be specifically cytotoxic to a range of tumour cell lines (Kessler et al. 2007; Rzeski et al. 2006), causing rapid increases in ROS production and a concomitant dissipation of mitochondrial membrane potential in a dose- and time-dependent manner, resulting in apoptosis (Liu et al. 2004). Betulinic acid was found to be nontoxic up to 500 mg/kg body weight in mice (Alakurtti et al. 2006). The target in mitochondria for betulinic acid related compounds has not been identified and their mechanism for initiating mitochondrial dysfunction leading to apoptosis remains unknown.

The sesquiterpene lactones are another group within the last class of mitocans encompassing a number of different naturally occurring structures that induce oxidative stress-mediated apoptosis in cancer cells acting in the micromolar range (Nakagawa et al. 2005) via loss of $\Delta\Psi_{m,i}$ and induction of ROS accumulation (Wen et al. 2002; Kim et al. 2005; Steele et al. 2006). They are also believed to target tumour stem cells while not affecting normal cells although their mechanism is presently unclear (Guzman et al. 2005).

Conclusions and Further Perspectives

As a greater understanding arises of the key differences between mitochondria from normal vs. malignant cells, we will gain in the ability to selectively target and kill tumours by activating apoptosis. Mitocans are providing a renewed impetus in the fight against cancer with their reduced level of side effects owing to their greater target selectivity. It is still too early in the clinical testing phases to decide whether mitocans will prove to be effective anticancer agents, although preliminary data indicates a strong likelihood of success. As a general rule, mitocans act by disrupting mitochondrial function in cancer cells, particularly of the OXPHOS system and the electron transport chain, causing overload reactions, ROS by-products, modification of thiol groups on key REDOX proteins and inhibition of pro-survival functions. The net effect is to activate apoptotic signal pathways in cancer cells. As anticancer drugs, the mitocans offer opportunities for additive or synergistic effects when used in combination with existing chemotherapeutic approaches, improving clinical outcomes. In addition, mitocans are providing approaches for directly attacking tumour stem cells. The goal is now in sight when we will be able to induce the clonal depletion and exhaustion of the progenitor cell populations, leading to cancer cures with mitocan therapy.

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References

Adam-Vizi, V. and Chinopoulos, C. 2006. Bioenergetics and the formation of mitochondrial reactive oxygen species. Trends Pharmacol Sci 27:639–645.

Alakurtti, S., Makela, T., Koskimies, S. and Yli-Kauhaluoma, J. 2006. Pharmacological properties of the ubiquitous natural product betulin. Eur J Pharm Sci 29:1–13.

Albores, R., Neafsey, E.J., Drucker, G., Fields, J.Z. and Collins, M.A. 1990. Mitochondrial respiratory inhibition by *N*-methylated -carboline derivatives structurally resembling *N*-methyl-4-phenylpyridine. Proc Natl Acad Sci USA 87:9368–9372.

Andreyev, A.Y., Kushnareva, Y.E. and Starkov, A.A. 2005. Mitochondrial metabolism of reactive oxygen species. Biochemistry (Moscow) 70:200–214.

Antignani, A. and Youle, R.J. 2006. How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane? Curr Opin Cell Biol 18:685–689.

Antonsson, B., Montessuit, S., Sanchez, B. and Martinou, J.C. 2001. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem 276:11615–11623.

Arner, E.S. and Holmgren, A. 2006. The thioredoxin system in cancer. Semin Cancer Biol 16:420–426.

Bacsi, A., Woodberry, M., Widger, W., Papaconstantinou, J., Mitra, S., Peterson, J.W. and Boldogh, I. 2006. Localization of superoxide anion production to mitochondrial electron transport chain in 3-NPA-treated cells. Mitochondrion 6:235–244.

Baggetto, L.G. 1992. Deviant energetic metabolism of glycolytic cancer cells. Biochimie 74:959–974.

Baggetto, L.G. and Lehninger, A.L. 1987. Isolated tumoral pyruvate dehydrogenase can synthesize acetoin which inhibits pyruvate oxidation as well as other aldehydes. Biochem Biophys Res Commun 145:153–159.

Baggetto, L.G. and Testa-Parussini, R. 1990. Role of acetoin on the regulation of intermediate metabolism of Ehrlich ascites tumor mitochondria: its contribution to membrane cholesterol enrichment modifying passive proton permeability. Arch Biochem Biophys 283:241–248.

Baggetto, L.G., Clottes, E. and Vial, C. 1992. Low mitochondrial proton leak due to high membrane cholesterol content and cytosolic creatine kinase as two features of the deviant bioenergetics of Ehrlich and AS30-D tumor cells. Cancer Res 52:4935–4941.

Bell, E.L. and Chandel, N.S. 2007. Mitochondrial oxygen sensing: regulation of hypoxia-inducible factor by mitochondrial generated reactive oxygen species. Essays Biochem 43:17–28.

Belogrudov, G.I. 2002. Factor B is essential for ATP synthesis by mitochondria. Arch Biochem Biophys 406:271–274.

Belogrudov, G.I. 2006. Bovine factor B: cloning, expression, and characterization. Arch Biochem Biophys 451:68–78.

Belogrudov, G.I. and Hatefi, Y. 2002. Factor B and the mitochondrial ATP synthase complex. J Biol Chem 277:6097–6103.

Belzacq, A.S., El Hamel, C., Vieira, H.L., Cohen, I., Haouzi, D., Metivier, D., Marchetti, P., Brenner, C. and Kroemer, G. 2001. Adenine nucleotide translocator mediates the mitochondrial membrane permeabilization induced by lonidamine, arsenite and CD437. Oncogene 20:7579–7587.

Bernal, S.D., Lampidis, T.J., Summerhayes, I.C. and Chen, L.B. 1982. Rhodamine-123 selectively reduces clonal growth of carcinoma cells in vitro. Science 218:1117–1119.

Bernal, S.D., Lampidis, T.J., McIsaac, R.M. and Chen, L.B. 1983. Anticarcinoma activity in vivo of rhodamine 123, a mitochondrial-specific dye. Science 222:169–172.

Berndt, C., Lillig, C.H. and Holmgren, A. 2007. Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system. Am J Physiol 292:H1227–H1236.

Bowman, E.J. and Bowman, B.J. 2005. V-ATPases as drug targets. J Bioenerg Biomembr 37:431–435.

Bowman, E.J., Gustafson, K.R., Bowman, B.J. and Boyd, M.R. 2003. Identification of a new chondropsin class of antitumor compound that selectively inhibits V-ATPases. J Biol Chem 278:44147–44152.

Brahimi-Horn, M.C., Chiche, J. and Pouyssegur, J. 2007. Hypoxia signalling controls metabolic demand. Curr Opin Cell Biol 19:223–229.

Britten, C.D., Rowinsky, E.K., Baker, S.D., Weiss, G.R., Smith, L., Stephenson, J., Rothenberg, M., Smetzer, L., Cramer, J., Collins, W., Von Hoff, D.D. and Eckhardt, S.G. 2000. A phase I and pharmacokinetic study of the mitochondrial-specific rhodacyanine dye analog MKT 077. Clin Cancer Res 6:42–49.

Brookes, P.S. 2005. Mitochondrial H+ leak and ROS generation: an odd couple. 2005. Free Radic Biol Med 38:12–23.

Brooks, G.A. 1998. Mammalian fuel utilization during sustained exercise. Comp Biochem Physiol B120:89–107.

Brooks, G.A. 2000. Intra- and extra-cellular lactate shuttles. Med Sci Sports Exer 32:790–799.

Brooks, G.A. 2002a. Lactate shuttle - between but not within cells? J Physiol 541:333.

Brooks, G.A. 2002b. Lactate shuttles in nature. Biochem Soc Trans 30:258–264.

Brooks, G.A., Brown, M.A., Butz, C.E., Sicurello, J.P. and Dubouchaud, H. 1999a. Cardiac and skeletal muscle mitochondria have a monocarboxylate transporter MCT1. J Appl Physiol 87:1713–1718.

Brooks, G.A., Dubouchaud, H., Brown, M., Sicurello, J.P. and Butz, C.E. 1999b. Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. Proc Natl Acad Sci USA 96:1129–1134.

Brown, J.M. and Giaccia, A.J. 1998. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 58:1408–1416.

Bustamante, E., Morris, H.P. and Pedersen, P.L. 1981. Energy metabolism of tumor cells. Requirement for a form of hexokinase with a propensity for mitochondrial binding. J Biol Chem 256:8699–8704.

Campian, J.L., Gao, X., Qian, M. and Eaton, J.W. 2007. Cytochrome C oxidase activity and oxygen tolerance. J Biol Chem 282:12430–12438.

Cantley, L.C. 2002. The phosphoinositide 3-kinase pathway. Science 296:1655–1657.

Cape, J.L., Bowman, M.K. and Kramer, D.M. 2007. A semiquinone intermediate generated at the Qo site of the cytochrome bc1 complex: importance for the Q-cycle and superoxide production. Proc Natl Acad Sci USA 104:7887–7892.

Capuano, F., Guerrieri, F. and Papa, S. 1997. Oxidative phosphorylation enzymes in normal and neoplastic cell growth. J Bioenerg Biomembr 29:379–384.

Capuano, F., Varone, D., D'Eri, N., Russo, E., Tommasi, S., Montemurro, S., Prete, F. and Papa, S. 1996. Oxidative phosphorylation and F0F1 ATP synthase activity of human hepatocellular carcinoma. Biochem Mol Biol Int 38:1013–1022.

Cardone, R.A., Casavola, V. and Reshkin, S.J. 2005. The role of disturbed pH dynamics and the Na +/H + exchanger in metastasis. Nat Rev Cancer 5:786–795.

Cartron, P.F., Arokium, H., Oliver, L., Meflah, K., Manon, S. and Vallette, F.M. 2005. Distinct domains control the addressing and the insertion of Bax into mitochondria. J Biol Chem 280:10587–10598.

Chandel, N.S., McClintock, D.S., Feliciano, C.E., Wood, T.M., Melendez, J.A., Rodriguez, A.M., and Schumacker, P.T. 2000. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1 during hypoxia: a mechanism of O2 sensing. J Biol Chem 275:25130–25138.

Chang, B.S., Minn, A.J., Muchmore, S.W., Fesik, S.W., Thompson, C.B. 1997. Identification of a novel regulatory domain in Bcl-xL and Bcl-2. EMBO J 16:968–977.

Chang, K.N., Lee, T.C., Tam, M.F., Chen, Y.C., Lee, L.W., Lee, S.Y., Lin, P.J. and Huang, R.N. 2003. Identification of galectin I and thioredoxin peroxidase II as two arsenic-binding proteins in Chinese hamster ovary cells. Biochem J 371:495–503.

Chang, T.S., Cho, C.S., Park, S., Yu, S., Kang, S.W. and Rhee, S.G. 2004. Peroxiredoxin III, a mitochondrion-specific peroxidase, regulates apoptotic signaling by mitochondria. J Biol Chem 279:41975–41984.

Chen, L.B. 1988. Mitochondrial membrane potential in living cells. Annu Rev Cell Biol 4:155–181.

Connor, K.M., Subbaram, S., Regan, K.J., Nelson, K.K., Mazurkiewicz, J.E., Bartholomew, P.J., Aplin, A.E., Tai, Y.T., Aguirre-Ghiso, J., Flores, S.C. and Melendez, J.A. 2005. Mitochondrial H2O2 regulates the angiogenic phenotype via PTEN oxidation. J Biol Chem 280:16916–16924.

Costantini, P., Chernyak, B.V., Petronilli, V. and Bernardi, P. 1996. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J Biol Chem 271:6746–6751.

Costantini, P., Belzacq, A.S., Vieira, H.L., Larochette, N., de Pablo, M.A., Zamzami, N., Susin, S. A., Brenner, C. and Kroemer, G. 2000. Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. Oncogene 19:307–314.

Cuezva, J.M., Ostronoff, L.K., Ricart, J., Lopez de Heredia, M., Di Ligero, C.M. and Izquierdo, J. M. 1997. Mitochondrial biogenesis in the liver during development and oncogenesis. J Bioenerg Biomembr 29:365–377.

Dahout-Gonzalez, C., Nury, H., Trezeguet, V., Lauquin, G.J., Pebay-Peyroula, E. and Brandolin, G. 2006. Molecular, functional, and pathological aspects of the mitochondrial ADP/ATP carrier. Physiology 21:242–249.

D'Alessio, M., De Nicola, M., Coppola, S., Gualandi, G., Pugliese, L., Cerella, C., Cristofanon, S., Civitareale, P., Ciriolo, M.R., Bergamaschi, A., Magrini, A. and Ghibelli, L. 2005. Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis. FASEB J 19:1504–1506.

Dalgard, C.L., Lu, H., Mohyeldin, A. and Verma, A. 2004. Endogenous 2-oxoacids differentially regulate expression of oxygen sensors. Biochem J 380:419–424.

Davey, G.P., Tipton, K.F. and Murphy, M.P. 1992. Uptake and accumulation of 1-methyl-4-phenylpyridinium by rat liver mitochondria measured using an ion-selective electrode. Biochem J 288:439–443.

Davis, S., Weiss, M.J., Wong, J.R., Lampidis, T.J. and Chen, L.B. 1985. Mitochondrial and plasma membrane potentials cause unusual accumulation and retention of rhodamine 123 by human breast adenocarcinoma-derived MCF-7 cells. J Biol Chem 260:13844–13850.

Degli-Esposti, M. and Dive, C. 2003. Mitochondrial membrane permeabilisation by Bax/Bak. Biochem Biophys Res Commun 304:455–461.

Deitrich, R.A., Petersen, D. and Vasiliou, V. 2007. Removal of acetaldehyde from the body. Novartis Found Symp 285:23–40.

Dilda, P.J., Decollogne, S., Rossiter-Thornton, M. and Hogg, P.J. 2005. Para to ortho repositioning of the arsenical moiety of the angiogenesis inhibitor 4-(*N*-(*S*-glutathionylacetyl)amino) phenylarsenoxide results in a markedly increased cellular accumulation and antiproliferative activity. Cancer Res 65:11729–11734.

Don, A.S., Kisker, O., Dilda, P., Donoghue, N., Zhao, X., Decollogne, S., Creighton, B., Flynn, E., Folkman, J. and Hogg, P.J. 2003. A peptide trivalent arsenical inhibits tumor angiogenesis by perturbing mitochondrial function in angiogenic endothelial cells. Cancer Cell 3:497–509.

Dong, L.F., Low, P., Dyason, J., Wang, X.F., Prochazka, L., Witting, P.K., Freeman, R., Swettenham, E., Valis, K., Liu, J., Zobalova, R., Turanek, J., Spitz, D.R., Domann, F.E., Scheffler, I.E., Ralph, S.J. and Neuzil, J. 2008. α-Tocopheryl succinate induces apoptosis by targeting biquinone-binding sites in mitochondrial respiratory complex II. Oncogene 27:4324–4335.

Dong, L.F., Swettenham, E., Eliasson, J., Wang, X.F., Gold, M., Medunic, Y., Stantic, M., Low, P., Prochazka, L., Witting, P.K., Turanek, J., Akporiaye, E.T., Ralph, S.J. and Neuzil, J. 2007. Vitamin E analogs inhibit angiogenesis by selective apoptosis induction in proliferating endothelial cells: The role of oxidative stress. Cancer Res 67:11906–11913.

Dyall, S.D., Agius, S.C., De Marcos Lousa, C., Trezeguet, V. and Tokatlidis, K. 2003. The dynamic dimerization of the yeast ADP/ATP carrier in the inner mitochondrial membrane is affected by conserved cysteine residues. J Biol Chem 278:26757–26764.

Ellerby, H.M., Arap, W., Ellerby, L.M., Kain, R., Andrusiak, R., Rio, G.D., Krajewski, S., Lombardo, C.R., Rao, R., Ruoslahti, E., Bredesen, D.E. and Pasqualini, R. 1999. Anti-cancer activity of targeted pro-apoptotic peptides. Nat Med 5:1032–1038.

Fantin, V.R., Berardi, M.J., Scorrano, L., Korsmeyer, S.J. and Leder, P. 2002. A novel mitochondriotoxic small molecule that selectively inhibits tumor cell growth. Cancer Cell 2:29–42.

Fantin, V.R., Berardi, M.J., Babbe, H., Michelman, M.V., Manning, C.M. and Leder, P. 2005. A bifunctional targeted peptide that blocks HER-2 tyrosine kinase and disables mitochondrial function in HER-2-positive carcinoma cells. Cancer Res 65:6891–6900.

Firth, J.D., Ebert, B.L. and Ratcliffe, P.J. 1995. Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. J Biol Chem 270:21021–21027.

Fomenko, D.E. and Gladyshev, V.N. 2003. Identity and functions of CxxC-derived motifs. Biochemistry 42:11214–11225.

Fomenko, D.E., Xing, W., Adair, B.M., Thomas, D.J. and Gladyshev, V.N. 2007. High-throughput identification of catalytic redox-active cysteine residues. Science 315:387–389.

Fukuda, R., Zhang, H., Kim, J.W., Shimoda, L., Dang, C.V. and Semenza, G.L. 2007. HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. Cell 129:111–122.

Gamboa-Vujicic, G., Emma, D.A., Liao, S.Y., Fuchtner, C. and Manetta, A. 1993. Toxicity of the mitochondrial poison dequalinium chloride in a murine model system. J Pharm Sci 82:231–235.

Garattini, E., Gianni, M. and Terao, M. 2004. Retinoid related molecules an emerging class of apoptotic agents with promising therapeutic potential in oncology: pharmacological activity and mechanisms of action. Curr Pharm Des 10:433–448.

Gerweck, L.E. 2000. The pH difference between tumor and normal tissue offers a tumor specific target for the treatment of cancer. Drug Resist Updat 3:49–50.

Gerweck, L.E., Vijayappa, S. and Kozin, S. 2006. Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics. Mol Cancer Ther 5:1275–1279.

Gincel, D., Zaid, H. and Shoshan-Barmatz, V. 2001. Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function. Biochem J 358:147–155.

Gledhill, J.R. and Walker, J.E. 2005. Inhibition sites in F1-ATPase from bovine heart mitochondria. Biochem J 386:591–598.

Gosslau, A., Chen, M., Ho, C.T. and Chen, K.Y. 2005. A methoxy derivative of resveratrol analogue selectively induced activation of the mitochondrial apoptotic pathway in transformed fibroblasts. Br J Cancer 92:513–521.

Gottlieb, E. and Tomlinson, I.P. 2005. Mitochondrial tumour suppressors: a genetic and biochemical update. Nat Rev Cancer 5:857–866.

Guzman, M.L., Rossi, R.M., Karnischky, L., Li, X., Peterson, D.R., Howard, D.S. and Jordan, C.T. 2005. The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells. Blood 105:4163–4169.

Hail, N. Jr, Kim, H.J. and Lotan, R. 2006. Mechanisms of fenretinide-induced apoptosis. Apoptosis 11:1677–1694.

Han, D., Williams, E. and Cadenas, E. 2001. Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. Biochem J 353 (Pt 2):411–416.

Han, D., Antunes, F., Daneri, F. and Cadenas, E. 2002. Mitochondrial superoxide anion production and release into intermembrane space. Methods Enzymol 349:271–280.

Han, D., Antunes, F., Canali, R., Rettori, D. and Cadenas, E. 2003. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. J Biol Chem 278:5557–5563.

Hansen, J.M., Zhang, H. and Jones, D.P. 2006. Differential oxidation of thioredoxin-1, thioredoxin-2, and glutathione by metal ions. Free Radic Biol Med 40:138–145.

Harguindey, S., Orive, G., Luis Pedraz, J., Paradiso, A. and Reshkin, S.J. 2005. The role of pH dynamics and the Na+/H+ antiporter in the etiopathogenesis and treatment of cancer. Two faces of the same coin - one single nature. Biochim Biophys Acta 1756:1–24.

Hirano, S., Kobayashi, Y., Hayakawa, T., Cui, X., Yamamoto, M., Kanno, S. and Shraim, A. 2005. Accumulation and toxicity of monophenyl arsenicals in rat endothelial cells. Arch Toxicol 79:54–61.

Hsieh, A.C. and Moasser, M.M. 2007. Targeting HER proteins in cancer therapy and the role of the non-target HER3. Br J Cancer 97:453–457.

Inarrea, P., Moini, H., Han, D., Rettori, D., Aguilo, I., Alava, M.A., Iturralde, M. and Cadenas, E. 2007. Mitochondrial respiratory chain and thioredoxin reductase regulate intermembrane Cu, Zn-superoxide dismutase activity: implications for mitochondrial energy metabolism and apoptosis. Biochem J 405:173–179.

Izumi, H., Torigoe, T., Ishiguchi, H., Uramoto, H., Yoshida, Y., Tanabe, M., Ise, T., Murakami, T., Yoshida, T., Nomoto, M. and Kohno, K. 2003. Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. Cancer Treat Rev 29:541–549.

Inarrea, P., Moini, H., Rettori, D., Han, D., Martinez, J., Garcia, I., Fernandez-Vizarra, E., Iturralde, M. and Cadenas, E. 2005. Redox activation of mitochondrial intermembrane space Cu,Zn-superoxide dismutase. Biochem J 387:203–209.

James, M.L., Selleri, S. and Kassiou, M. 2006. Development of ligands for the peripheral benzodiazepine receptor. Curr Med Chem 13:1991–2001.

Jones, L.W., Narayan, K.S., Shapiro, C.E. and Sweatman, T.W. 2005. Rhodamine-123: therapy for hormone refractory prostate cancer, a phase I clinical trial. J Chemother 17:435–440.
Joshi, S. and Hughes, J.B. 1981. Inhibition of coupling factor B activity by cadmium ion, arsenite-2,3-dimercaptopropanol, and phenylarsine oxide, and preferential reactivation by dithiols. J Biol Chem 256:11112–11116.
Kagan, V.E., Tyurin, V.A., Jiang, J., Tyurina, Y.Y., Ritov, V.B., Amoscato, A.A., Osipov, A.N., Belikova, N.A., Kapralov, A.A., Kini, V., Vlasova, I.I., Zhao, Q., Zou, M., Di, P., Svistunenko, D.A., Kurnikov, I.V. and Borisenko, G.G. 2005. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nat Chem Biol 1:223–232.
Kallio, A., Zheng, A., Dahllund, J., Heiskanen, K.M. and Harkonen, P. 2005. Role of mitochondria in tamoxifen-induced rapid death of MCF-7 breast cancer cells. Apoptosis 10:1395–1410.
Kessler, J.H., Mullauer, F.B., de Roo, G.M. and Medema, J.P. 2007. Broad in vitro efficacy of plant-derived betulinic acid against cell lines derived from the most prevalent human cancer types. Cancer Lett 251:132–145.
Kim, J.H., Liu, L., Lee, S.O., Kim, Y.T., You, K.R. and Kim, D.G. 2005. Susceptibility of cholangiocarcinoma cells to parthenolide-induced apoptosis. Cancer Res 65:6312–6320.
Kim, J.-W., Gao, P. and Dang, C.V. 2007. Effects of hypoxia on tumor metabolism. Cancer Metastasis Rev 27:291–298.
King, A., Selak, M.A. and Gottlieb, E. 2006. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. Oncogene 25:4675–4682.
Knowles, H.J. and Harris, A.L. 2001. Hypoxia and oxidative stress in breast cancer. Hypoxia and tumourigenesis. Breast Cancer Res 3:318–322.
Ko, Y.H., Smith, B.L., Wang, Y., Pomper, M.G., Rini, D.A., Torbenson, M.S., Hullihen, J. and Pedersen, P.L. 2004. Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. Biochem Biophys Res Commun 324:269–275.
Koivunen, P., Tiainen, P., Hyvarinen, J., Williams, K.E., Sormunen, R., Klaus, S.J., Kivirikko, K.I. and Myllyharju, J. 2007. An endoplasmic reticulum transmembrane prolyl 4-hydroxylase is induced by hypoxia and acts on HIF-α . J Biol Chem. 282:30544–30552.
Koobs, D.H. 1972. Phosphate mediation of the Crabtree and Pasteur effects. Does the change in energy metabolism enhance the potential for malignancy? Science 178:127–133.
Koukourakis, M.I., Giatromanolaki, A., Simopoulos, C., Polychronidis, A. and Sivridis, E. 2005. Lactate dehydrogenase 5 (LDH5) relates to up-regulated hypoxia inducible factor pathway and metastasis in colorectal cancer. Clin Exp Metastasis 22:25–30.
Kozin, S.V., Shkarin, P. and Gerweck, L.E. (2001) The cell transmembrane pH gradient in tumors enhances cytotoxicity of specific weak acid chemotherapeutics. Cancer Res 61:4740–4743.
Kwon, J., Lee, S.R., Yang, K.S., Ahn, Y., Kim, Y.J., Stadtman, E.R. and Rhee, S.G. 2004. Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. Proc Natl Acad Sci USA 101:16419–16424.
Lampidis, T.J., Bernal, S.D., Summerhayes, I.C. and Chen, L.B. 1983. Selective toxicity of rhodamine 123 in carcinoma cells in vitro. Cancer Res 43:716–720.
Lampidis, T.J., Hasin, Y., Weiss, M.J. and Chen, L.B. 1985. Selective killing of carcinoma cells "in vitro" by lipophilic-cationic compounds: a cellular basis. Biomed Pharmacother 39:220–226.
Larochette, N., Decaudin, D., Jacotot, E., Brenner, C., Marzo, I., Susin, S.A., Zamzami, N., Xie, Z., Reed, J. and Kroemer, G. 1999. Arsenite induces apoptosis via a direct effect on the mitochondrial permeability transition pore. Exp Cell Res 249:413–421.
Larsson, A. 1973. Thioredoxin reductase from rat liver. Eur J Biochem 35:346–349.
Lee, A.H. and Tannock, I.F. 1998. Heterogeneity of intracellular pH and of mechanisms that regulate intracellular pH in populations of cultured cells. Cancer Res 58:1901–1908.
Lee, S.R., Yang, K.S., Kwon, J., Lee, C., Jeong, W. and Rhee, S.G. 2002. Reversible inactivation of the tumor suppressor PTEN by H2O2. J Biol Chem 277:20336–20342.
Lenaz, G. 2001. The mitochondrial production of reactive oxygen species: Mechanisms and implications in human pathology. IUBMB Life 52:159–164.

Liu, W.K., Ho, J.C., Cheung, F.W., Liu, B.P., Ye, W.C. and Che, C.T. 2004. Apoptotic activity of betulinic acid derivatives on murine melanoma B16 cell line. Eur J Pharmacol 498:71–78.

Liu, J., Rone, M.B. and Papadopoulos, V. 2006. Protein-protein interactions mediate mitochondrial cholesterol transport and steroid biosynthesis. J Biol Chem 281:38879–38893.

Lu, H., Dalgard, C.L., Mohyeldin, A., McFate, T., Tait, A.S. and Verma, A. 2005. Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. J Biol Chem 280:41928–41939.

Lu, X., Qin, W., Li, J., Tan, N., Pan, D., Zhang, H., Xie, L., Yao, G., Shu, H., Yao, M., Wan, D., Gu, J. and Yang, S. (2005) The growth and metastasis of human hepatocellular carcinoma xenografts are inhibited by small interfering RNA targeting to the subunit ATP6L of proton pump. Cancer Res 65:6843–6849.

Maaser, K., Sutter, A.P. and Scherubl, H. 2005. Mechanisms of mitochondrial apoptosis induced by peripheral benzodiazepine receptor ligands in human colorectal cancer cells. Biochem Biophys Res Commun 332:646–652.

Machida, K., Ohta, Y. and Osada, H. 2006. Suppression of apoptosis by cyclophilin D via stabilization of hexokinase II mitochondrial binding in cancer cells. J Biol Chem 281:14314–14320.

MacKenzie, E.D., Selak, M.A., Tennant, D.A., Payne, L.J., Crosby, S., Frederiksen, C.M., Watson, D.G. and Gottlieb, E. 2007. Cell-permeating -ketoglutarate derivatives alleviate pseudohypoxia in succinate dehydrogenase-deficient cells. Mol Cell Biol 27:3282–3289.

Madesh, M. and Hajnoczky, G. 2001. VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release. J Cell Biol 155:1003–1015.

Makin, G.W., Corfe, B.M., Griffiths, G.J., Thistlethwaite, A., Hickman, J.A. and Dive, C. 2001. Damage-induced Bax N-terminal change, translocation to mitochondria and formation of Bax dimers/complexes occur regardless of cell fate. EMBO J 20:6306–6315.

Manning, B.D. and Cantley, L.C. 2007. AKT/PKB signaling: navigating downstream. Cell 129:1261–1274.

Mathupala, S.P., Ko, Y.H. and Pedersen, P.L. 2006. Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. Oncogene 25:4777–4786.

McLennan, H.R. and Degli Esposti, M. 2000. The contribution of mitochondrial respiratory complexes to the production of reactive oxygen species. J Bioenerg Biomembr 32:153–162.

McStay, G.P., Clarke, S.J. and Halestrap, A.P. 2002. Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. Biochem J 367:541–548.

Mikhailov, V., Mikhailova, M., Pulkrabek, D.J., Dong, Z., Venkatachalam, M.A. and Saikumar, P. 2001. Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. J Biol Chem 276:18361–18374.

Modica-Napolitano, J.S. and Aprille, J.R. 1987. Basis for the selective cytotoxicity of rhodamine 123. Cancer Res 47:4361–4365.

Modica-Napolitano, J.S. and Aprille, J.R. 2001. Delocalized lipophilic cations selectively target the mitochondria of carcinoma cells. Adv Drug Deliv Rev 49:63–70.

Modica-Napolitano, J.S. and Singh, K. 2002. Mitochondria as targets for detection and treatment of cancer. Expert Rev Mol Med 2002:1–19.

Monkkonen, H., Auriola, S., Lehenkari, P., Kellinsalmi, M., Hassinen, I.E., Vepsalainen, J. and Monkkonen, J. 2006. A new endogenous ATP analog (ApppI) inhibits the mitochondrial adenine nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-containing bisphosphonates. Br J Pharmacol 147:437–445.

Nakagawa, Y., Iinuma, M., Matsuura, N., Yi, K., Naoi, M., Nakayama, T., Nozawa, Y. and Akao, Y. 2005. A potent apoptosis-inducing activity of a sesquiterpene lactone, arucanolide, in HL60 cells: a crucial role of apoptosis-inducing factor. J Pharmacol Sci 97:242–252.

Nechushtan, A., Smith, C.L., Hsu, Y.T. and Youle, R.J. 1999. Conformation of the Bax C-terminus regulates subcellular location and cell death. EMBO J 18:2330–2341.

Neuzil, J., Zhao, M., Ostermann, G., Sticha, M., Gellert, N., Weber, C., Eaton, J.W. and Brunk, U. T. 2002. -Tocopheryl succinate, an agent with in vivo anti-tumour activity, induces apoptosis by causing lysosomal instability. Biochem J 362:709–715.

Neuzil, J., Wang, X.F., Dong, L.F., Low, P. and Ralph, S.J. 2006. Molecular mechanism of 'mitocan'-induced apoptosis in cancer cells epitomizes the multiple roles of reactive oxygen species and Bcl-2 family proteins. FEBS Lett 580:5125–5129.

Neuzil, J., Dyason, J.C., Freeman, R., Dong, L.F., Prochazka, L., Wang, X.F., Scheffler, I., Ralph, S.J. 2007. Mitocans as anti-cancer agents targeting mitochondria: lessons from studies with vitamin E analogues, inhibitors of complex II. J Bioenerg Biomembr 39:65–72.

Nonn, L., Berggren, M. and Powis, G. 2003. Increased expression of mitochondrial peroxiredoxin-3 (thioredoxin peroxidase-2) protects cancer cells against hypoxia and drug-induced hydrogen peroxide-dependent apoptosis. Mol Cancer Res 1:682–689.

Nury, H., Dahout-Gonzalez, C., Trezeguet, V., Lauquin, G.J., Brandolin, G. and Pebay-Peyroula, E. 2006. Relations between structure and function of the mitochondrial ADP/ATP carrier. Annu Rev Biochem 75:713–741.

O'Hare, T., Corbin, A.S. and Druker, B.J. 2006. Targeted CML therapy: controlling drug resistance, seeking cure. Curr Opin Genet Dev 16:92–99.

Ohnishi, T. and Salerno, J.C. 2005. Conformation-driven and semiquinone-gated proton-pump mechanism in the NADH-ubiquinone oxidoreductase (complex I). FEBS Lett 579:4555–4561.

O'Neill, J., Manion, M., Schwartz, P. and Hockenbery, D.M. 2004. Promises and challenges of targeting Bcl-2 anti-apoptotic proteins for cancer therapy. Biochim Biophys Acta 1705:43–51.

Ott, M., Robertson, J.D., Gogvadze, V., Zhivotovsky, B. and Orrenius, S. 2002. Cytochrome c release from mitochondria proceeds by a two-step process. Proc Natl Acad Sci USA 99:1259–1263.

Ott, M., Gogvadze, V., Orrenius, S. and Zhivotovsky, B. 2007. Mitochondria, oxidative stress and cell death. Apoptosis 12:913–922.

Paddenberg, R., Ishaq, B., Goldenberg, A., Faulhammer, P., Rose, F., Weissmann, N., Braun-Dullaeus, R.C. and Kummer, W. 2003. Essential role of complex II of the respiratory chain in hypoxia-induced ROS generation in the pulmonary vasculature. Am J Physiol 284:L710–L719.

Pagliari, L.J., Kuwana, T., Bonzon, C., Newmeyer, D.D., Tu, S., Beere, H.M. and Green, D.R. 2005. The multidomain proapoptotic molecules Bax and Bak are directly activated by heat. Proc Natl Acad Sci USA 102:17975–17980.

Papadopoulos, V., Baraldi, M., Guilarte, T.R., Knudsen, T.B., Lacapere, J.J., Lindemann, P., Norenberg, M.D., Nutt, D., Weizman, A., Zhang, M.R. and Gavish, M. 2006. Translocator protein (18 kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol Sci 27:402–409.

Papandreou, I., Cairns, R.A., Fontana, L., Lim, A.L. and Denko, N.C. 2006. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab 3:187–197.

Parolin, M.L., Chesley, A., Matsos, M.P., Spriet, L.L., Jones, N.L. and Heigenhauser, G.J.F. 1999. Regulation of skeletal muscle glycogen phosphorylase and PDH during maximal intermittent exercise. Am J Physiol 277:E890–E900.

Pastorino, J.G., Shulga, N. and Hoek, J.B. 2002. Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. J Biol Chem 277:7610–7618.

Pedersen, P.L. and Morris, H.P. 1974. Uncoupler stimulated adenosine triphosphatase activity. Deficiency in intact mitochondria from Morris hepatomas and ascites tumor cells. J Biol Chem 249:3327–3334.

Pelicano, H., Feng, L., Zhou, Y., Carew, J.S., Hileman, E.O., Plunkett, W., Keating, M.J. and Huang, P. 2003. Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. J Biol Chem 278:37832–37839.

Pelicano, H., Xu, R.H., Du, M., Feng, L., Sasaki, R., Carew, J.S., Hu, Y., Ramdas, L., Hu, L., Keating, M.J., Zhang, W., Plunkett, W. and Huang, P. 2006. Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox-mediated mechanism. J Cell Biol 175:913–923.

Perier, C., Tieu, K., Guegan, C., Caspersen, C., Jackson-Lewis, V., Carelli, V., Martinuzzi, A., Hirano, M., Przedborski, S. and Vila, M. 2005. Complex I deficiency primes Bax-dependent neuronal apoptosis through mitochondrial oxidative damage. Proc Natl Acad Sci USA 102:19126–19131.

Petrosillo, G., Ruggiero, F.M., Pistolese, M. and Paradies, G. 2001. Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis. FEBS Lett 509:435–438.

Pollard, P.J., Briere, J.J., Alam, N.A. et al. 2005. Accumulation of Krebs cycle intermediates and over-expression of HIF1 in tumours which result from germline FH and SDH mutations. Hum Mol Genet 14:2231–2239.

Pretner, E., Amri, H., Li, W., Brown, R., Lin, C.S., Makariou, E., Defeudis, F.V., Drieu, K. and Papadopoulos, V. 2006. Cancer-related overexpression of the peripheral-type benzodiazepine receptor and cytostatic anticancer effects of Ginkgo biloba extract (EGb 761). Anticancer Res 26:9–22.

Ralph, S.J., Low, P., Dong, L., Lawen, A. and Neuzil, J. 2006. Mitocans: mitochondrial targeted anti-cancer drugs as improved therapies and related patent documents. Recent Patents Anti-cancer Drug Discovery 1:327–346.

Ralph, S.J., Dyason, J.C., Freeman, R., Dong, L.F., Prochazka, L., Wang, X.F., Scheffler, I.E. and Neuzil, J. 2007. Mitocans as anti-cancer agents targeting mitochondria: Lessons from studies with vitamin E analogues, inhibitors of complex II. J Bioenerg Biomembr 39:65–72.

Robey, R.B. and Hay, N. 2006. Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt. Oncogene 25:4683–4696.

Roche, T.E. and Hiromasa, Y. 2007. Pyruvate dehydrogenase kinase regulatory mechanisms and inhibition in treating diabetes, heart ischemia, and cancer. Cell Mol Life Sci 64:830–849.

Rzeski, W., Stepulak, A., Szymanski, M., Sifringer, M., Kaczor, J., Wejksza, K., Zdzisinska, B. and Kandefer-Szerszen, M. 2006. Betulinic acid decreases expression of bcl-2 and cyclin D1, inhibits proliferation, migration and induces apoptosis in cancer cells. Naunyn Schmiedebergs Arch Pharmacol 374:11–20.

Sahara, N., Takeshita, A., Kobayashi, M., Shigeno, K., Nakamura, S., Shinjo, K., Naito, K., Maekawa, M., Horii, T., Ohnishi, K., Kitamura, K., Naoe, T., Hayash, H. and Ohno, R. 2004. Phenylarsine oxide (PAO) more intensely induces apoptosis in acute promyelocytic leukemia and As2O3-resistant APL cell lines than As2O3 by activating the mitochondrial pathway. Leuk Lymphoma 45:987–995.

Sanborn, B.M., Felberg, N.T. and Hollocher, T.C. 1971. The inactivation of succinate dehydrogenase by bromopyruvate. Biochim Biophys Acta 227:219–231.

Sareen, D., van Ginkel, P.R., Takach, J.C., Mohiuddin, A., Darjatmoko, S.R., Albert, D.M. and Polans, A.S. 2006. Mitochondria as the primary target of resveratrol-induced apoptosis in human retinoblastoma cells. Invest Ophthalmol Vis Sci 47:3708–3716.

Selak,M.A., Armour, S.M., MacKenzie, E.D., Boulahbel, H., Watson, D.G., Mansfield, K.D., Pan, Y., Simon, M.C., Thompson, C.B. and Gottlieb, E. 2005. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- prolyl hydroxylase. Cancer Cell 7:77–85.

Semenza, G.L. 2007. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. Biochem J 405:1–9.

Sennoune, S.R., Luo, D. and Martinez-Zaguilan, R. 2004. Plasmalemmal vacuolar-type H + -ATPase in cancer biology. Cell Biochem Biophys 40:185–206.

Shafir, I., Feng, W. and Shoshan-Barmataz, V. 1998. Voltage-dependent anion channel proteins in synaptosomes of the torpedo electric organ: immunolocalization, purification, and characterization. J Bioenerg Biomembr 30:499–510.

Shiau, C.W., Huang, J.W., Wang, D.S., Weng, J.R., Yang, C.C., Lin, C.H., Li, C. and Chen, C.S. 2006. Tocopheryl succinate induces apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 function. J Biol Chem 281:11819–11825.

Shoshan-Barmatz, V., Israelson, A., Brdiczka, D. and Sheu, S.S. 2006. The voltage-dependent anion channel (VDAC): function in intracellular signalling, cell life and cell death. Curr Pharm Des 12:2249–2270.

Skarsgard, L.D., Chaplin, D.J., Wilson, D.J., Skwarchuk, M.W., Vinczan, A. and Kristl, J. 1992. The effect of hypoxia and low pH on the cytotoxicity of chlorambucil. Int J Radiat Oncol Biol Phys 22:737–741.

Slepkov, E.R., Rainey, J.K., Sykes, B.D. and Fliegel, L. 2007. Structural and functional analysis of the Na+/H+ exchanger. Biochem J 401:623–633.

Slot, J.W., Geuze, H.J., Freeman, B.A. and Crapo, J.D. 1986. Intracellular localization of the copper-zinc and manganese superoxide dismutases in rat liver parenchymal cells. Lab Invest 55:363–371.

Smaili, S.S., Hsu, Y.T., Sanders, K.M., Russell, J.T. and Youle, R.J. 2001. Bax translocation to mitochondria subsequent to a rapid loss of mitochondrial membrane potential. Cell Death Differ 8:909–920.

Smith, R.A., Porteous, C.M., Gane, A.M. and Murphy, M.P. 2003. Delivery of bioactive molecules to mitochondria in vivo. Proc Natl Acad Sci USA 100:5407–5412.

Stankiewicz, A.R., Lachapelle, G., Foo, C.P., Radicioni, S.M. and Mosser, D.D. 2005. Hsp70 inhibits heat-induced apoptosis upstream of mitochondria by preventing Bax translocation. J Biol Chem 280:38729–38739.

Steele, A.J., Jones, D.T., Ganeshaguru, K., Duke, V.M., Yogashangary, B.C., North, J.M., Lowdell, M.W., Kottaridis, P.D., Mehta, A.B., Prentice, A.G., Hoffbrand, A.V. and Wickremasinghe, R.G. 2006. The sesquiterpene lactone parthenolide induces selective apoptosis of B-chronic lymphocytic leukemia cells in vitro. Leukemia 20:1073–1079.

Summerhayes, I.C., Lampidis, T.J., Bernal, S.D., Nadakavukaren, J.J., Nadakavukaren, K.K., Shepherd, E.L. and Chen, L.B. 1982. Unusual retention of rhodamine 123 by mitochondria in muscle and carcinoma cells. Proc Natl Acad Sci USA 79:5292–5296.

Suzuki, M., Youle, R.J. and Tjandra, N. 2000. Structure of Bax: coregulation of dimer formation and intracellular localization. Cell 103:645–654.

Tan, C., Dlugosz, P.J., Peng, J., Zhang, Z., Lapolla, S.M., Plafker, S.M., Andrews, D.W. and Lin, J. 2006. Auto-activation of the apoptosis protein BAX increases mitochondrial membrane permeability and is inhibited by BCL-2. J Biol Chem 281:14764–14775.

Tinhofer, I., Bernhard, D., Senfter, M., Anether, G., Loeffler, M., Kroemer, G., Kofler, R., Csordas, A. and Greil, R. 2001. Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by Bcl-2. FASEB J 15:1613–1615.

Trachootham, D., Zhou, Y., Zhang, H., Demizu, Y., Chen, Z., Pelicano, H., Chiao, P.J., Achanta, G., Arlinghaus, R.B., Liu, J. and Huang, P. 2006. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by -phenylethyl isothiocyanate. Cancer Cell 10:241–252.

Trapp, S. and Horobin, R.W. 2005. A predictive model for the selective accumulation of chemicals in tumor cells. Eur Biophys J 34:959–966.

Turrens, J.F. 1997. Superoxide production by the mitochondrial respiratory chain. Biosci Rep 17:3–8.

Turrens, J.F., Alexandrem, A. and Lehninger, A.L. 1985. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch Biochem Biophys 237:408–414.

van Delft, M.F., Wei, A.H., Mason, K.D., Vandenberg, C.J., Chen, L., Czabotar, P.E., Willis, S.N., Scott, C.L., Day, C.L., Cory, S., Adams, J.M., Roberts, A.W. and Huang, D.C. 2006. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 10:389–399.

Vaupel, P. and Mayer, A. 2007. Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 26:225–239.

Verrier, F., Deniaud, A., Lebras, M., Metivier, D., Kroemer, G., Mignotte, B., Jan, G. and Brenner, C. 2004. Dynamic evolution of the adenine nucleotide translocase interactome during chemotherapy-induced apoptosis. Oncogene 23:8049–8064.

Vivanco, I. and Sawyers, C.L. 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2:489–501.

Vyssokikh, M., Zorova, L., Zorov, D., Heimlich, G., Jurgensmeier, J., Schreiner, D. and Brdiczka, D. 2004. The intra-mitochondrial cytochrome c distribution varies correlated to the formation of a complex between VDAC and the adenine nucleotide translocase: this affects Bax-dependent cytochrome c release. Biochim Biophys Acta 1644:27–36.

Walenta, S. and Mueller-Klieser, W.F. 2004. Lactate: mirror and motor of tumor malignancy. Semin Radiat Oncol 14:267–274.

Wallace, D.C. 2005. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet 39:359–407.

Wang, K., Gross, A., Waksman, G. and Korsmeyer, S.J. 1998. Mutagenesis of the BH3 domain of BAX identifies residues critical for dimerization and killing. Mol Cell Biol 18:6083–6089.

Warburg, O. 1956. On the origin of cancer cells. Science 123:309–314.

Warburg, O., Wind, F. and Neglers, E. 1930. On the metabolism of tumors in the body. In Metabolism of Tumors, O. Warburg (ed.). pp 254-270. London: Arnold Constable and Co. Press.

Wen, J., You, K.R., Lee, S.Y., Song, C.H. and Kim, D.G. 2002. Oxidative stress-mediated apoptosis. The anticancer effect of the sesquiterpene lactone parthenolide. J Biol Chem 277:38954–38964.

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. and Youle, R.J. 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol 139:1281–1292.

Wu, X., Senechal, K., Neshat, M.S., Whang, Y.E. and Sawyers, C.L. 1998. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. Proc Natl Acad Sci USA 95:15587–15591.

Xu, Y., Salerno, J.C., Wei, Y.H. and King, T.E. 1987. Stabilized ubisemiquinone in reconstituted succinate ubiquinone reductase. Biochem Biophys Res Commun 144:315–322.

Xiao, D., Lew, K.L., Zeng, Y., Xiao, H., Marynowski, S.W., Dhir, R. and Singh, S.V. 2006. Phenethyl isothiocyanate-induced apoptosis in PC-3 human prostate cancer cells is mediated by reactive oxygen species-dependent disruption of the mitochondrial membrane potential. Carcinogenesis 27:2223–2234.

Yethon, J.A., Epand, R.F., Leber, B., Epand, R.M. and Andrews, D.W. 2003. Interaction with a membrane surface triggers a reversible conformational change in Bax normally associated with induction of apoptosis. J Biol Chem 278:48935–48941.

Yu, C.A., Nagoaka, S., Yu, L. and King, T.E. 1980. Evidence of ubisemiquinone radicals in electron transfer at the cytochromes b and c1 region of the cardiac respiratory chain. Arch Biochem Biophys 204:59–70.

Zalk, R., Israelson, A., Garty, E.S., Azoulay-Zohar, H. and Shoshan-Barmatz, V. 2005. Oligomeric states of the voltage-dependent anion channel and cytochrome c release from mitochondria. Biochem J 386:73–83.

Zha, A., Aime-Sempe, C., Sato, T. and Reed, J.C. 1996. Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. J Biol Chem 271:7440–7444.

Zhang, R., Al-Lamki, R., Bai, L., Streb, J.W., Miano, J.M., Bradley, J. and Min, W. 2004. Thioredoxin-2 inhibits mitochondria-located ASK1-mediated apoptosis in a JNK-independent manner. Circ Res 94:1483–1491.

Zhang, Z.Y., Davis, J.P. and Van Etten, R.L. 1992. Covalent modification and active site-directed inactivation of a low molecular weight phosphotyrosyl protein phosphatase. Biochemistry 31:1701–1711.

Zhang, Z., Lapolla, S.M., Annis, M.G., Truscott, M., Roberts, G.J., Miao, Y., Shao, Y., Tan, C., Peng, J., Johnson, A.E., Zhang, X.C., Andrews, D.W. and Lin, J. 2004. Bcl-2 homodimerization involves two distinct binding surfaces, a topographic arrangement that provides an effective mechanism for Bcl-2 to capture activated Bax. J Biol Chem 279:43920–43928.

Zhao, K., Zhao, G.M., Wu, D., Soong, Y., Birk, A.V., Schiller, P.W. and Szeto, H.H. 2004. Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. J Biol Chem 279:34682–34690.

Zheng, Y., Shi, Y., Tian, C., Jiang, C., Jin, H., Chen, J., Almasan, A., Tang, H. and Chen, Q. 2004. Essential role of the voltage-dependent anion channel (VDAC) in mitochondrial permeability transition pore opening and cytochrome c release induced by arsenic trioxide. Oncogene 23:1239–1247.

Zhivotovsky, B., Orrenius, S., Brustugun, O.T. and Doskeland, S.O. 1998. Injected cytochrome c induces apoptosis. Nature 391:449–450.

Zhou, L., Jing, Y., Styblo, M., Chen, Z. and Waxman, S. 2005. Glutathione-*S*-transferase pi inhibits As2O3-induced apoptosis in lymphoma cells: involvement of hydrogen peroxide catabolism. Blood 105:1198–1203.

Zini, R., Morin, C., Bertelli, A., Bertelli, A.A. and Tillement, J.P. 1999. Effects of resveratrol on the rat brain respiratory chain. Drugs Exp Clin Res 25:87–97.

Zuckerbraun, B.S., Chin, B.Y., Bilban, M., de Costa d'Avila, J., Rao, J., Billiar, T.R. and Otterbein, L.E. 2007. Carbon monoxide signals via inhibition of cytochrome c oxidase and generation of mitochondrial reactive oxygen species. FASEB J 21:1099–1106.

Zunino, S.J. and Storms, D.H. 2006. Resveratrol-induced apoptosis is enhanced in acute lymphoblastic leukemia cells by modulation of the mitochondrial permeability transition pore. Cancer Lett 240:123–134.

Reversing the Warburg Effect: Metabolic Modulation as a Novel Cancer Therapy

Gopinath Sutendra and Evangelos D. Michelakis

Introduction

Despite the research efforts and enormous investments from the industry and the public, oncology has an impressively poor success rate in the clinical development of effective investigational drugs; less than a third of that in cardiovascular or infectious diseases (Kamb et al. 2007). Traditionally, oncology drug development has focused *essential* targets (i.e. essential for the survival of all dividing cells). This approach suffers from low selectivity and thus narrow therapeutic windows. In contrast, more selectivity is achieved by targeting nonessential targets, but as expected, this approach suffers from poor efficacy. It is extremely rare to find a target that is essential only to cancer cells; the dependence of chronic myelogenous leukemia cells on Ableson kinase is induced by a chromosomal translocation only in the malignant cells, making the efficacy and selectivity of imatinib, an *exception* in experimental oncology (Kamb et al. 2007).

The biggest challenges in cancer therapeutics are the remarkable heterogeneity and adoptability of cancer cells. The molecular characteristics of the same type of tumor between two patients are often dissimilar. Even within the same tumor, significant heterogeneity often exists with different parts of the tumor, showing dependence on different signaling pathways; glioblastoma is a notable example. The view that "there are many different types of cancers" is shared by the majority of scientists and oncologists. This has led to the realization that specific drugs have to be developed and tested for specific tumors and effects on one might not necessarily be relevant to another tumor.

One way that the problem of heterogeneity of "proximal" molecular pathways in cancer can be addressed is by targeting more "distal" pathways that integrate several proximal signals, for as long as they remain unique and necessary for the survival of cancer and not normal cells. The unique metabolism of most solid

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tumors integrates many proximal pathways and is associated with a remodeling of mitochondria (where the regulation of energy production and apoptosis converge), expressed as a glycolytic phenotype and a strong resistance to apoptosis. This cancer-specific remodeling can be reversed by dichloroacetate (DCA), a mitochondria-targeting small molecule, that penetrates most tissues after oral administration (Bonnet et al. 2007a; Pan and Mak 2007). The molecular and direct metabolic response to DCA can be followed by measuring glucose uptake in tumors by Positron Emission Tomography (PET) imaging, noninvasively and prospectively. The preclinical work on DCA (showing effectiveness in a variety of tumors and relatively low toxicity), its structure (a very small molecule), the low price (it is a generic drug) and the fact that DCA has already been used in humans for more than 30 years (Stacpoole 1969, 1989; Stacpoole et al. 2003), provide a strong rationale for rapid clinical development. Such metabolic strategies might be able to shift the paradigm of experimental therapeutics in oncology.

Here, we discuss the scientific rationale as well as practical points that might become important in the clinical development of metabolic modulators like DCA.

Metabolism in the Evolutionary and Genetic Models of Cancer Development

Glucose oxidation (GO) is the optimal way of producing energy from carbohydrates and requires intact mitochondria and oxygen. Typically, tissues switch to the less efficient cytoplasmic glycolysis (Gly) when mitochondria are dysfunctional or when oxygen levels are low (hypoxia). Intriguingly, most cancers are characterized by aerobic glycolysis, i.e., they use glycolysis for energy production, although oxygen is present. In 1930, Warburg first observed this (i.e. the Warburg effect) and suggested that this is a result of mitochondrial dysfunction, preventing the mitochondria-based GO (Warburg 1930). Because GO is far more effective in generating ATP compared with Gly (36 vs. 2 ATP generated per glucose molecule), cancer cells upregulate glucose receptors and significantly increase glucose uptake in an attempt to "catch up". PET imaging has now confirmed that most solid tumors have significantly increased glucose uptake and metabolism, compared with non-cancerous tissues (Gambhir 2002). This bioenergetic difference between cancer and normal cells might offer a very selective therapeutic target. However, this area of experimental oncology has remained controversial; the glycolytic profile has traditionally been viewed as a *result* of cancer progression, not a *cause* and therefore the interest in targeting tumor metabolism has been low.

In addition, at first glance this unique metabolic profile in cancer is difficult to understand, at least teleologically. First, why would these highly proliferating and energy-demanding cells rely on Gly rather than the much more efficient GO? Second, Gly results in significant lactic acidosis, which might cause significant toxicity to the surrounding tissues and the cancer cells themselves. Recent advances

have caused a rekindling of the metabolic hypothesis of cancer, suggesting that these facts are not as conflicting as they appear at first (Gatenby and Gillies 2004):

Glycolysis and the "evolutionary model" of cancer growth: Gatenby and Gillies recently proposed that since early carcinogenesis often occurs in a hypoxic microenvironment, the transformed cells have to rely on anaerobic Gly (Gatenby and Gillies 2004). Hypoxia-inducible factor (HIF) is activated by hypoxia and it has been shown to induce the expression of several glucose transporters and most of the enzymes required for Gly (Robey et al. 2005; Semenza et al. 2001). Among others, HIF induces the expression of pyruvate dehydrogenase kinase (PDK) (Kim et al. 2006), a gate-keeping enzyme that regulates the flux of carbohydrates (pyruvate) into the mitochondria. In the presence of activated PDK, pyruvate dehydrogenase (PDH) is inhibited, limiting the entry of pyruvate into the mitochondria, thus limiting GO. Therefore activated PDK promotes completion of Gly in the cytoplasm with metabolism of pyruvate into lactate, whereas inhibited PDK ensures an efficient coupling between Gly and GO and completion of pyruvate metabolism into the mitochondria.

Initially, tumors compensate by increasing glucose uptake into the cells. Furthermore, Gatenby and Gillies list several mechanisms through which the lactic acidosis facilitates tumor growth: breakdown of extra-cellular matrix allowing expansion, increased cell mobility/metastatic potential, and (along with HIF) activation of angiogenesis (Gatenby and Gillies 2004). The tumors eventually become vascularized and are not significantly hypoxic anymore (although some tumors remain hypoxic at the core because the quality of the neovessel formation is poor (Neri and Bicknell 2005)). However, the glycolytic profile persists (i.e., aerobic glycolysis). This suggests that the (initially adaptive) metabolic remodeling offers a survival/proliferating advantage to cancer cells. Recent evidence suggests that transformation to a glycolytic phenotype offers resistance to apoptosis (Plas and Thompson 2002) (Kim and Dang 2005, 2006).

Glycolysis and the genetic model of cancer: it is now well-recognized that several of the enzymes involved in glycolysis are also important regulators of apoptosis and gene transcription, suggesting that links between metabolic sensors, cell death, and gene transcription are established directly through the enzymes that control metabolism (Kim and Dang 2005). For example, hexokinase activation leads to a significant suppression of apoptosis; activated hexokinase translocates from the cytoplasm to the mitochondrial membranes where it interacts with and suppresses several key components of mitochondria-dependent apoptosis (Kim and Dang 2005; Pastorino and Hoek 2003). It is therefore not surprising that hexokinase is upregulated and activated in many cancers (Kim and Dang 2005; Pastorino and Hoek 2003). How does this occur? The promoter of hexokinase contains both p53 and HIF response elements and both mutated p53 and activated HIF increase hexokinase expression (Mathupala et al. 1997, 2001). In another example, the oncogenic protein Akt (Osaki et al. 2004; Thompson and Thompson 2004) induces a glycolytic metabolic profile via a several mechanisms, including an increase in expression and activity of hexokinase (Cross et al. 1995; Elstrom et al. 2004; Gottlob et al. 2001; Osaki et al. 2004). The gene that normally antagonizes Akt,

PTEN, is mutated (loss of function mutation) in a large number of cancers. Recent data revealed even more links between p53 and metabolism: p53 regulates the expression of a critical enzyme of Gly via the production of TIGAR and is also directly regulating the expression of a subunit of *cytochrome c oxidase* (Zhou et al. 2003), an important component of complex IV of the electron transport chain in mitochondria (reviewed in Pan and Mak 2007). In other words, the most common molecular abnormality in cancer, i.e., the loss of p53 function, induces metabolic and mitochondrial changes, compatible with the glycolytic phenotype. Another example of a classic oncogene that induces the same metabolic phenotype is c-myc; this transcription factor increases the expression of many enzymes of Gly (Kim and Dang 2006).

The central role of metabolism in both carcinogenesis (the genetic model) and cancer growth (the evolutionary model) suggests that it might be centrally involved in the pathogenesis of cancer and that it is not simply a "result" of carcinogenesis. Although this metabolic phenotype might not directly and by itself induce malignancy, it certainly "facilitates" carcinogenesis and cancer growth (Kim and Dang 2006). In addition, this metabolic signature is the common denominator of multiple and diverse "proximal" pathways; which means that if it is therapeutically targeted it might offer selectivity to cancer and effectiveness in many different cancers.

A Mitochondria-K^+ Channel Axis and the Regulation of Apoptosis (Fig. 1)

Mitochondria are major and direct regulators of apoptosis (Green and Kroemer 2004). As discussed later, the level of mitochondria activity is directly related with the threshold for apoptosis and can also be easily studied by measuring the mitochondrial membrane potential with easy and standard imaging techniques.

GO starts by the formation of acetyl-CoA, which then enters the Krebs' cycle, from which the electron donors NADH and FADH are produced and "donate" their electron in the electron transport chain (ETC). The electrons are flowing down a redox gradient from complex I to complex IV, with molecular O_2 being the final acceptor. As the electrons flow, H^+ are generated and their transfer out of the internal mitochondrial membrane creates the mitochondrial membrane potential ($\Delta\Psi_m$), which is quite negative (about -200 mV). At the same time, the flow of electrons also causes production of superoxide, mainly at complex I and III. The amount of superoxide produced varies according to the amount of electrons flowing, i.e., according to respiration. The ATP synthase at the end of the ETC brings H^+ back into the inner membrane (thus using the stored energy of the $\Delta\Psi_m$) and phosphorylates ADP, producing ATP. Thereby respiration is coupled to oxidative phosphorylation; thus $\Delta\Psi_m$ *is an index of activity in the mitochondria* (Duchen 1999). The fact that the mitochondria are the most negative organelles in the cell is taken advantage by positive fluorescent dyes (like TMRM) that can selectively accumulate in the mitochondria; since the higher the $\Delta\Psi_m$ the higher the fluorescence, these dyes allow for study of the $\Delta\Psi_m$, a surrogate for mitochondrial function.

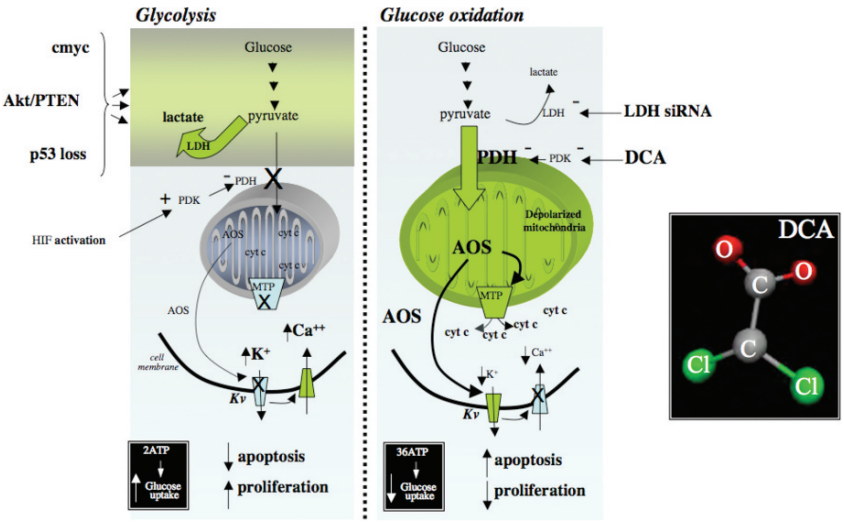

Fig. 1 A glycolytic environment is associated with an antiapoptotic and pro-proliferative state, characterizing most solid tumors. Increased entry of pyruvate into the mitochondria by either DCA or inhibition of LDH promotes glucose oxidation, increased apoptosis, and decreased proliferation and tumor growth (see text for discussion)

On the basis of the discussions made so far, the flow and the process of fuel (i.e. the metabolism) can regulate the $\Delta\Psi_m$. $\Delta\Psi_m$ in turn can regulate several important functions that regulate numerous signaling mechanisms. First, $\Delta\Psi_m$ regulates the opening of the *voltage-sensitive* mitochondrial transition pore (MTP), a mega-channel that includes the voltage-dependent anion channel (VDAC). Significant mitochondrial depolarization (decreased $\Delta\Psi_m$) leads to opening of the MTP (Zamzami and Kroemer 2001), which results in the efflux of several pro-apoptotic mediators that induce apoptosis either by activating caspases (i.e., cytochorme c) or by directly lysing DNA (i.e., apoptosis inducing factor, which translocates into the nucleus). In that sense, hyperpolarization of the mitochondria (high $\Delta\Psi_m$) away from the threshold of MTP opening, suggests a state of *resistance to apoptosis*. Second, since the production of superoxide is directly associated to the flux of electrons through the ETC, then $\Delta\Psi_m$ is associated with the production of stable AOS that provide signaling downstream from mitochondria. For example the relatively stable H_2O_2 (produced by superoxide in the presence of the intramitochondrial manganese superoxide dismutase) activates the redox-sensitive voltage-gated K^+ channels (Kv) (Michelakis et al. 2004; Weir et al. 2005). Opening of Kv channels results in efflux of K^+, down its concentration gradient. Because K^+ exerts a tonic inhibition of caspases in numerous cell types (Remillard and Yuan 2004), this Kv opening further promotes apoptosis. Third, a shift of energy production from the mitochondria (GO) to the cytoplasm (Gly) is associated with a decrease in glycogen synthase kinase 3 (GSK-3), an inhibitor of hexokinase; in addition to its role in Gly, activated hexokinase is translocated to the mitochondrial membrane,

where it interacts and inhibits the VDAC, resulting in mitochondrial hyperpolarization (the inhibition of VDAC prevents the efflux of anions down an electrical gradient, thus increasing intra-mitochondrial anions) (Pastorino and Hoek 2003; Pastorino et al. 2002, 2005); by phosphorylating VDAC and disrupting hexokinase, GSK-3 depolarizes the mitochondria and induces apoptosis.

In summary, there are multiple parallel ways by which metabolism (a glycolytic environment) induces and regulates a mitochondrial remodeling (hyperpolarization) that is associated with decreased levels of mitochondrial AOS and inhibited Kv channels, all promoting resistance to apoptosis. It is therefore not surprising that, as described later, mitochondria of cancer cells have been known to be hyperpolarized compared with that of noncancer cells for more than 25 years. What is surprising is that this has not been linked to apoptosis resistance in cancer.

Mitochondria in Cancer

Can the unique metabolic phenotype in cancer (aerobic glycolysis) be translated into a unique mitochondrial remodeling? In other words, are remodeled mitochondria a universal marker of cancer? If true, this would have important implications since mitochondria function can be easily and rapidly studied in biological specimens (using rhodamine-based dyes, like TMRM) and since mitochondria function is linked to apoptosis, it might be another marker of cancer aggressiveness. It is intriguing that since the 1980s Dr Chen at the Dana-Farber Institute had shown that the majority of cancer cells have hyperpolarized mitochondria compared with noncancer cells (Chen 1988). He wrote: “The results of a six-year systematic study overwhelmingly indicate that all normal epithelial cells tested have low mitochondrial membrane potential, hence, low rhodamine123 uptake and retention. In contrast, screenings of 200 cell lines/types derived from tumors of kidney, ovary, pancreas, lung, adrenal cortex, skin, breast, prostate, cervix, vulva, colon, liver, testis, esophagus, trachea, and tongue show that a great majority of adenocarcinoma, transitional cell carcinoma, squamous cell carcinoma, and melanoma have high rhodamine123 uptake and retention; Chen also stated that the most notable exceptions included oat cell and large cell adenocarcinoma of the lung, leukemias, lymphomas, neuroblastomas, and osteosarcomas (Chen 1988). He made these observations using rhodamine123, a positively-changed dye used to study mitochondrial membrane potential. The difference in mitochondrial potential between cancer and normal cells was at least 80 mV. Physiologically this is a huge difference as a 60 mV potential across a 5 nm membrane is equivalent to a potential of 120,000 V across 1 cm. Although the reason for these studies was the potential ability of these dyes to be used as vehicles for the selective delivery of drugs in cancer cells, the relevance of the mitochondrial membrane potential to apoptosis threshold and its association with the metabolic changes in cancer cells were not appreciated by the scientific community. The reason for this hyperpolarization is unknown and likely multifactorial. As discussed earlier, an intriguing

scenario suggests that in a glycolyic environment hexokinase translocates to the mitochondria, binds to and inhibits VDAC, resulting in hyperpolarization (Pastorino et al. 2005).

We confirmed Chen's findings and found that reversal of mitochondrial hyperpolarization in cancer cells by DCA reversed the resistance to apoptosis and inhibited tumor growth:

DCA in Cancer: Preclinical Work

We found that three different cancer cell lines (nonsmall-cell lung cancer, breast cancer, and glioblastoma) had hyperpolarized mitochondria, compared with noncancer cell lines (Bonnet et al. 2007a). This was associated with suppressed levels of mitochondria-derived AOS and decreased activity and expression of Kv channels. The suppressed K^+ channels also contributed to the increased intracellular calcium levels in the cancer, compared to the noncancer cells (Bonnet et al. 2007a). This is because the inhibition of K^+ channels leads to depolarization of the plasma membrane, which in turn activates the voltage-dependent calcium channels, allowing influx of calcium into the cell. Lastly, the Ca^{++}-sensitive transcription factor NFAT (nuclear factor of activated T cells (Macian 2005)) was also active (i.e. nuclear) in the cancer cells. NFAT is a transcription factor that has been shown to regulate the expression of a number of metabolic enzymes and to increase the levels of the antiapoptotic bcl-2 and decrease the levels of the Kv channel Kv1.5 (Bonnet et al. 2007b) (a Kv channel particularly important in apoptosis (Remillard and Yuan 2004)). All of these features could be secondary to a suppressed mitochondrial activity: decrease entry of pyruvate would eventually result in decrease flux of electrons in the ETC and therefore decreased AOS production, closing of the existing redox-sensitive Kv channels and increased intracellular Ca^{++}, activating NFAT. The decreased AOS could also contribute to closure of the redox-sensitive MTP and mitochondrial hyperpolarization. The decreased entry of pyruvate into the mitochondria (and therefore the decreased GO) would result in Gly, all the elements of which are known to be upregulated in cancer. Increased hexokinase levels would contribute to the hyperpolarization of the mitochondria.

DCA depolarized cancer mitochondria, returning the membrane potential towards the levels of the noncancer cells, without affecting the mitochondria of noncancerous cells (Fig. 2). Remarkably, all of the above features of the cancer cells were "normalized" following the mitochondrial depolarization: GO increased, AOS increased, NFAT was inactivated and function/expression of Kv1.5 channels was increased. Most importantly, apoptosis was induced in the cancer cells with both cyt c and apoptosis inducing factor efflux from the mitochondria. This resulted in a decrease in tumor growth both in vitro and in vivo in xenotransplant models (Fig. 3). The mechanism of DCA was, as predicted, inhibition of PDK2 (the most ubiquitously expressed isoenzyme and the one with the

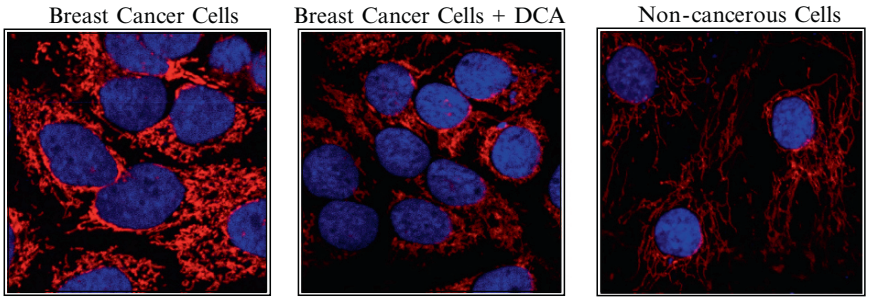

Fig. 2 Breast cancer mitochondria are hyperpolarized, compared with noncancerous cells. DCA depolarizes mitochondria in mammary carcinoma cells (See Color Insert)

highest sensitivity to DCA) since PDK2 siRNA completely mimicked DCA and addition of DCA to the PDK2 siRNA did not have any additional effects.

The fact that GO (which only takes place within mitochondria) was increased by DCA clearly shows that cancer mitochondria are functional and not irreversibly damaged as previously thought. This further raises the intriguing possibility that in cancer there is an active and dynamic suppression of mitochondrial function in order to also suppress apoptosis. "Refueling" of mitochondria by driving pyruvate inside them reverses this suppression, reactivates the apoptotic machinery (increased AOS, decreased $\Delta\Psi_m$, open MTP) and unlocks the cancer cells from a state of apoptosis resistance.

It is important here to clarify that simply inhibiting Gly (particularly at proximal stages), will not reactivate mitochondria. It will also be toxic to several noncancerous tissues that depend on Gly for energy production. Inhibiting Gly (which has previously been tested as a potential treatment for cancer) typically results in necrosis, not apoptosis, since apoptosis is an energy-consuming process, requiring active mitochondria. The "trick" is to enhance the Gly to GO coupling, not just inhibit Gly. One of the ways that this can happen is by activating PDH by DCA, or by inhibiting LDH (lactate dehydrogenase): they will both result in bringing pyruvate into the mitochondria and enhancing GO (Fig. 1). Inhibition of LDH-A by short hairpin RNAs, mimicked DCA: it increased mitochondrial activity, depolarized mitochondria, inducing apoptosis and decreasing cancer growth in vitro and in vivo in xenotransplant models (Fantin et al. 2006).

DCA in the Treatment of Congenital Mitochondrial Diseases

DCA is a small molecule of 150 daltons that is completely ionized at biological pH. The small size of the molecule explains in part the high bioavailability of this drug and the fact that it can penetrate otherwise challenging for standard chemotherapy

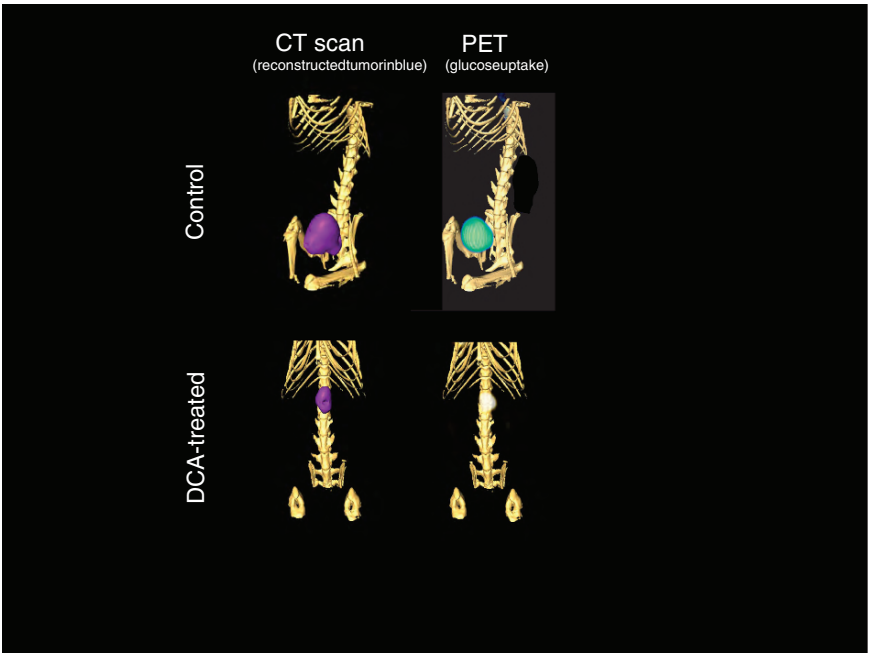

Fig. 3 Nonsmall-cell lung cancer cells were injected in the flank of nude rats. The rats are imaged with a rodent PET-CT (GammaMedica). Simultaneous CT and FDG-Glucose PET imaging shows that DCA therapy decreases both the size and the glucose uptake in the tumor (note that the intensity of the PET signal in the DCA treated rat is lower compared with that of the untreated control). The Warburg effect can also be appreciated, since the increased glucose uptake is confined within the tumor

areas, like the blood brain barrier. In vitro, DCA activates PDH by inhibition of PDK at concentration of 10–250 μM or 0.15–37.5 μg/mL in a dose-dependent fashion (Bersin and Stacpoole 1997; Wells et al. 1980). The crystal structure of human PDK2 bound with DCA is shown in Fig. 4: the carboxylate group of DCA forms a salt-bridge with Arg-154 while its two chlorine residues are deeply buried in a hydrophobic pocket (Knoechel et al. 2006).

A large number of patients (children and adults) have been exposed to DCA over the past 40 years. These include healthy volunteers and many diverse disease states. The idea behind using DCA in humans since its first description in 1969 (Stacpoole 1969) so far has been the alleviation of symptoms or the hemodynamic consequences that are directly related to the lactic acidosis complicating diverse diseases (e.g.: severe malaria, sepsis, congestive heart failure, burns, cirrhosis, liver transplantation, congenital mitochondrial diseases). Various single-arm and randomized trials of DCA given at doses ranging from 12.5 to 100 mg/kg/day orally or intravenously for various medical conditions that lead to acidosis have been reported (reviewed in (Stacpoole et al. 2003)). DCA was universally effective in lowering lactate levels, but (as expected) was not effective in altering the course of

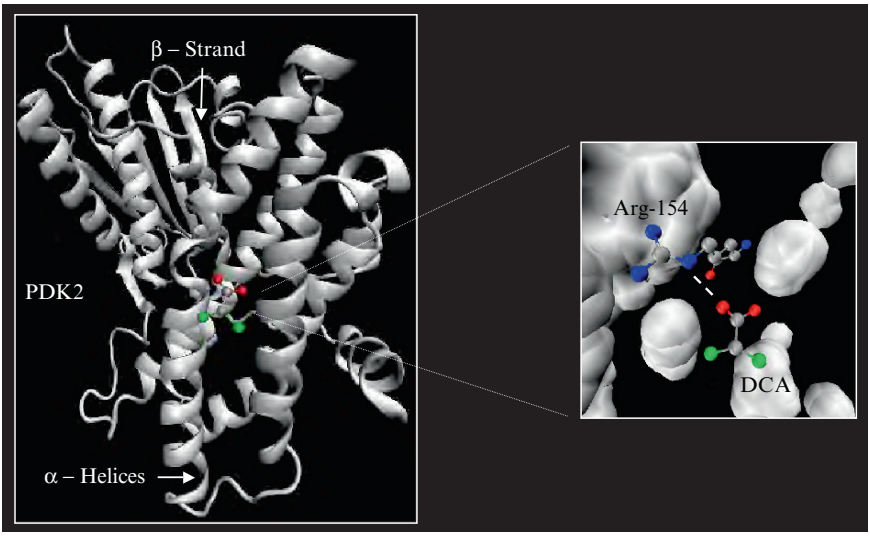

Fig. 4 Structure of human pyruvate dehydrogenase kinase 2 (PDK2) bound with dichloroacetate (DCA). Ribbon representation of PDK2 with DCA bound is shown. In the zoom, surface representation of PDK2 bound with DCA is shown. The carboxylate group of DCA forms a salt bridge (dashed white line) with Arginine 154 of PDK2. The structures were generated using visual molecular dynamics (VMD) version 1.8.6 with the coordinates acquired from Protein Data Bank (PDB#2BU8)

the primary disease (for example the sepsis) and therefore it did not alter the outcome of the primary disease.

The first two randomized control trials of chronic oral therapy with DCA in mitochondrial diseases were reported in 2006. In the first, a blinded randomized placebo-controlled study was performed with oral DCA administered at 25 mg/kg/day in 30 patients with MELAS syndrome (Mitochondrial myopathy, Encephalopathy, LActic acidosis, and Stroke-like episodes for 2 years. After an initial 30 patients were enrolled, most patients enrolled in the DCA arm developed symptomatic peripheral neuropathy, compared to 4 out of 15 in the placebo arm. This led to the termination of the study. Seventeen out of 19 patients had at least partial resolution of peripheral neurological symptoms by 9 months after discontinuation of DCA. No other toxicities were reported in the study. It is important to note that peripheral neuropathy frequently complicates (at varying preclinical or clinical degrees patients with MELAS due to primary or secondary effects on peripheral nerves; for example patients with MELAS also have diabetes and diabetes-related peripheral neuropathy).

At the same time, another placebo controlled double blinded study failed to sow any significant toxicity of DCA, including peripheral neuropathy. In that study only one out of 21 children with congenital lactic acidosis treated with DCA orally at 25 mg/kg/day for six months demonstrated mild peripheral neuropathy. Serial nerve conduction studies failed to demonstrate any difference in incidence of neuropathy

in the 2 arms (placebo vs DCA). Sleepiness and lethargy, muscular rigidity of the upper extremity and hand tremor were reported in 1 patient in each group (Stacpoole et al. 2006).

Therefore, the higher incidence of peripheral neuropathy in adult MELAS may be partly the result of the natural history of the disease, a result of associated conditions like diabetes mellitus; this toxicity might also be age-dependent. In summary, this is a potentially concerning side effect that if it occurs appears to be reversible. It is important to emphasize that peripheral neuropathy is a relatively common complication of standard chemotherapy, that often complicates chemotherapy agents that are considered to be well-tolerated overall.

DCA: Clinical Translation in Oncology

There is solid evidence in preclinical in vitro and in vivo models that DCA might be beneficial in human cancer. The concept is strengthened by the fact that LDH inhibition in mice with human cancer xenotransplants, mimicked DCA (Fantin et al. 2006). The induction of PDK by HIF (Kim et al. 2006) is an important finding particularly in the context that (at least in lung cancers) PDK is upregulated in cancer cells but not in the neighboring noncancerous fibroblasts in human biopsies (Koukourakis et al. 2005). Treatment with DCA in patients has provided ample clinical and mechanistic pharmacokinetic and toxicity data in randomized studies.

The realization that a diverse group of signaling pathways and oncogenes result in resistance to apoptosis and a glycolytic phenotype, the fact that the majority of carcinomas have hyperpolarized/remodeled mitochondria and have increased glucose uptake in PET, suggest that DCA might be effective in a large number of diverse tumors. However, direct evidence of anticancer effects of DCA has been published so far only in nonsmall-cell lung cancer, glioblastoma, and breast cancer. In addition, the lack of mitochondrial hyperpolarization in certain types of cancer including oat cell lung cancer, lymphomas, neuroblastomas, and certain types of sarcomas suggest that there will be exceptions. Cancers with limited or no meaningful therapeutic options like glioblastoma or lung cancer should be in top of the list of cancers to be studied.

Carefully performed phase I and II, dose-escalating trials are indicated in order to find the optimal dose of DCA in cancer. Clinical trials on DCA need to monitor neurotoxicity carefully, establishing clear dose-de-escalation or escape protocols; furthermore more work is needed in order to determine who is predisposed to this adverse effect, potentially through pharmacogenomic arms in early protocols.

There is preclinical evidence that DCA, as a single agent, might be effective in cancer; therefore clinical trials with DCA as a single agent or in direct comparison with other agents are indicated. However, since DCA “unlocks” cancer cells from a state of apoptosis resistance, DCA might be an attractive “apoptosis sensitizer”. In that sense, DCA will both precede and be given simultaneously with standard chemo or radiation therapy, in an attempt to increase the effectiveness, decrease the required

doses, and thus limit the toxicity of standard therapies. These "combination" trials are indicated after the first round of trials examining DCA as a single agent.

Funding for these trials will be a challenge for the academic community since DCA is a generic drug and industry support might be challenging. Fundraising from philanthropies might be possible to support early phase I–II or small phase III trials. However, if these trials suggest a favorable efficacy and toxicity, the public may be further motivated to directly fund these efforts and national cancer organizations like the NCI might be inspired to directly contribute to the design and structure of larger trials. It is important to note that even if DCA does not prove to be the "dawn of a new era" (Pan and Mak 2007), initiation and completion of clinical trials with a generic compound will be a task of tremendous symbolic and practical significance. In that sense, the clinical development of DCA, in addition to its scientific rationale, will be by itself another paradigm shift.

References

Bersin, R.M. and Stacpoole, P.W. (1997). Dichloroacetate as metabolic therapy for myocardial ischemia and failure. *Am Heart J* **134**, 841–55.

Bonnet, S., Archer, S.L., Allalunis-Turner, J., Haromy, A., Beaulieu, C., Thompson, R., Lee, C.T., Lopaschuk, G.D., Puttagunta, L., Bonnet, S., Harry, G., Hashimoto, K., Porter, C.J., Andrade, M.A., Thebaud, B. and Michelakis, E.D. (2007a). A mitochondria-K^+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell* **11**, 37–51.

Bonnet, S., Rochefort, G., Sutendra, G., Archer, S.L., Haromy, A., Webster, L., Hashimoto, K., Bonnet, S.N. and Michelakis, E.D. (2007b). The nuclear factor of activated T cells in pulmonary arterial hypertension can be therapeutically targeted. *Proc Natl Acad Sci USA* **104**, 11418–23.

Chen, L.B. (1988). Mitochondrial membrane potential in living cells. *Annu Rev Cell Biol* **4**, 155–81.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M. and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785–9.

Duchen, M.R. (1999). Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. *J Physiol* **516 (Pt 1)**, 1–17.

Elstrom, R.L., Bauer, D.E., Buzzai, M., Karnauskas, R., Harris, M.H., Plas, D.R., Zhuang, H., Cinalli, R.M., Alavi, A., Rudin, C.M. and Thompson, C.B. (2004). Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res* **64**, 3892–9.

Fantin, V.R., St-Pierre, J. and Leder, P. (2006). Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell* **9**, 425–34.

Gambhir, S.S. (2002). Molecular imaging of cancer with positron emission tomography. *Nat Rev Cancer* **2**, 683–93.

Gatenby, R.A. and Gillies, R.J. (2004). Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* **4**, 891–9.

Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R.B. and Hay, N. (2001). Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev* **15**, 1406–18.

Green, D.R. and Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science* **305**, 626–9.

Kamb, A., Wee, S. and Lengauer, C. (2007). Why is cancer drug discovery so difficult? *Nat Rev Drug Discov* **6**, 115–20.

Kim, J.W. and Dang, C.V. (2005). Multifaceted roles of glycolytic enzymes. *Trends Biochem Sci* **30**, 142–50.

Kim, J.W. and Dang, C.V. (2006). Cancer's molecular sweet tooth and the Warburg effect. *Cancer Res* **66**, 8927–30.

Kim, J.W., Tchernyshyov, I., Semenza, G.L. and Dang, C.V. (2006). HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* **3**, 177–85.

Knoechel, T.R., Tucker, A.D., Robinson, C.M., Phillips, C., Taylor, W., Bungay, P.J., Kasten, S. A., Roche, T.E. and Brown, D.G. (2006). Regulatory roles of the N-terminal domain based on crystal structures of human pyruvate dehydrogenase kinase 2 containing physiological and synthetic ligands. *Biochemistry* **45**, 402–15.

Koukourakis, M.I., Giatromanolaki, A., Sivridis, E., Gatter, K.C. and Harris, A.L. (2005). Pyruvate dehydrogenase and pyruvate dehydrogenase kinase expression in non small cell lung cancer and tumor-associated stroma. *Neoplasia* **7**, 1–6.

Macian, F. (2005). NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* **5**, 472–84.

Mathupala, S.P., Heese, C. and Pedersen, P.L. (1997). Glucose catabolism in cancer cells. The type II hexokinase promoter contains functionally active response elements for the tumor suppressor p53. *J Biol Chem* **272**, 22776–80.

Mathupala, S.P., Rempel, A. and Pedersen, P.L. (2001). Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. *J Biol Chem* **276**, 43407–12.

Michelakis, E.D., Thebaud, B., Weir, E.K. and Archer, S.L. (2004). Hypoxic pulmonary vasoconstriction: redox regulation of O2-sensitive K^+ channels by a mitochondrial O2-sensor in resistance artery smooth muscle cells. *J Mol Cell Cardiol* **37**, 1119–36.

Neri, D. and Bicknell, R. (2005). Tumour vascular targeting. *Nat Rev Cancer* **5**, 436–46.

Osaki, M., Oshimura, M. and Ito, H. (2004). PI3K-Akt pathway: its functions and alterations in human cancer. *Apoptosis* **9**, 667–76.

Pan, J.G. and Mak, T.W. (2007). Metabolic targeting as an anticancer strategy: dawn of a new era? *Sci STKE* 2007, pe14.

Pastorino, J.G. and Hoek, J.B. (2003). Hexokinase II: the integration of energy metabolism and control of apoptosis. *Curr Med Chem* **10**, 1535–51.

Pastorino, J.G., Shulga, N. and Hoek, J.B. (2002). Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. *J Biol Chem* **277**, 7610–8.

Pastorino, J.G., Hoek, J.B. and Shulga, N. (2005). Activation of glycogen synthase kinase 3beta disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity. *Cancer Res* **65**, 10545–54.

Plas, D.R. and Thompson, C.B. (2002). Cell metabolism in the regulation of programmed cell death. *Trends Endocrinol Metab* **13**, 75–8.

Remillard, C.V. and Yuan, J.X. (2004). Activation of K^+ channels: an essential pathway in programmed cell death. *Am J Physiol Lung Cell Mol Physiol* **286**, L49–L67.

Robey, I.F., Lien, A.D., Welsh, S.J., Baggett, B.K. and Gillies, R.J. (2005). Hypoxia-inducible factor-1alpha and the glycolytic phenotype in tumors. *Neoplasia* **7**, 324–30.

Semenza, G.L., Artemov, D., Bedi, A., Bhujwalla, Z., Chiles, K., Feldser, D., Laughner, E., Ravi, R., Simons, J., Taghavi, P. and Zhong, H. (2001). 'The metabolism of tumours': 70 years later. *Novartis Found Symp* **240**, 251–60; discussion 260–4.

Stacpoole, P.W. (1969). Review of the pharmacologic and therapeutic effects of diisopropylammonium dichloroacetate (DIPA). *J Clin Pharmacol J New Drugs* **9**, 282–91.

Stacpoole, P.W. (1989). The pharmacology of dichloroacetate. *Metabolism* **38**, 1124–44.

Stacpoole, P.W., Nagaraja, N.V. and Hutson, A.D. (2003). Efficacy of dichloroacetate as a lactate-lowering drug. *J Clin Pharmacol* **43**, 683–91.

Stacpoole, P.W., Kerr, D.S., Barnes, C., Bunch, S.T., Carney, P.R., Fennell, E.M., Felitsyn, N.M., Gilmore, R.L., Greer, M., Henderson, G.N., Hutson, A.D., Neiberger, R.E., O'Brien, R.G., Perkins, L.A., Quisling, R.G., Shroads, A.L., Shuster, J.J., Silverstein, J.H., Theriaque, D.W. and Valenstein, E. (2006). Controlled clinical trial of dichloroacetate for treatment of congenital lactic acidosis in children. *Pediatrics* **117**, 1519–31.

Thompson, J.E. and Thompson, C.B. (2004). Putting the rap on Akt. *J Clin Oncol*, 22, 4217-26.

Warburg, O. (1930). *Ueber den stoffwechsel der tumoren*. Constable: London.

Weir, E.K., Lopez-Barneo, J., Buckler, K.J. and Archer, S.L. (2005). Acute oxygen-sensing mechanisms. *N Engl J Med* **353**, 2042–55.

Wells, P.G., Moore, G.W., Rabin, D., Wilkinson, G.R., Oates, J.A. and Stacpoole, P.W. (1980). Metabolic effects and pharmacokinetics of intravenously administered dichloroacetate in humans. *Diabetologia* **19**, 109–13.

Zamzami, N. and Kroemer, G. (2001). The mitochondrion in apoptosis: how Pandora's box opens. *Nat Rev Mol Cell Biol* **2**, 67–71.

Zhou, S., Kachhap, S. and Singh, K.K. (2003). Mitochondrial impairment in p53-deficient human cancer cells. *Mutagenesis* **18**, 287–92.

Mitochondrial Nanotechnology for Cancer Therapy

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Introduction

The tight association of mitochondrial dysfunction with the pathogenesis of cancer has been the subject of preceding chapters in this book. It has been made evident that a rapidly growing insight into the link between cancer and mitochondrial functions is the basis for identifying new molecular sites at or inside mitochondria that potentially present novel drug targets for pharmacological intervention. Subsequently, the mitochondrial network as an emerging target of anticancer therapy has been the subject of another chapter in this book. The development of pharmacological agents specifically aimed at compromising the structural and functional integrity of mitochondria materializes as a new approach to combat cancer cell proliferation. Already, a large variety of chemically diverse small molecules have been demonstrated to have direct effects on mitochondrial morphology and functions, either at the DNA level or upon targeting proteins located in the inner or outer mitochondrial membranes (Costantini et al. 2000, Dias and Bailly 2005). The potential clinical success of any new experimental drug, however, is determined by its therapeutic efficiency. That is, the question has to be addressed whether and to what extent the drug is able to reach the site of the tumor while possibly avoiding healthy tissues, to what extent the drug is then able to enter the tumor cell, and finally, whether the drug is able to reach its actual intracellular molecular target.

This chapter discusses the pharmaceutical aspect of exploring mitochondria as a target for anticancer therapy. FDA-approved nanotechnologies for anticancer chemotherapy will be described briefly followed by a more extensive discussion of newly emerging nanotechnologies aimed at the specific delivery drugs to their intracellular molecular site of action.

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Nanotechnology and Cancer Therapy

Literally all cancer drugs are associated with dose-limiting toxicity. One of the major challenges in treating cancer is the lack of selectivity of the currently available cytotoxic agents. Many such agents fail to significantly distinguish between cancer cells and healthy cells. Consequently, systemic application of these drugs often causes severe side effects in other tissues (e.g., bone marrow suppression, cardiomyopathy, neurotoxicity), which greatly limits the maximal allowable dose of the drug, i.e., the therapeutic index is low. Another major issue in the treatment of cancer is multidrug resistance (MDR). There are multiple putative origins of MDR, but one of the most studied today is the efflux pump. The pump serves to remove drug molecules from the cell before they can act at their particular subcellular target inside the cell. Other forms of MDR are associated with the nucleus, including the DNA repair system. Both problems mentioned above, i.e., nonspecifity and MDR, can potentially be solved by developing highly selective tumor-specific drug delivery systems.

Progress in this direction has already been made with the development and subsequent FDA approval of Doxil®/Caelyx® and DaunoXome®. Doxil® is a poly(ethylene glycol)-grafted phospholipid vesicle of 100 nm diameter with encapsulated doxorubicin hydrochloride; DaunoXome® is daunorubicin encapsulated in vesicles 45 nm in size. Doxorubicin and daunorubicin containing phospholipid vesicles (“liposomes”) have been designed to circulate for an extended period of time in the blood system, which eventually results in an increased accumulation of the drug at sites of vascular damage, i.e., at solid tumors, a phenomena known as enhanced permeability and retention (EPR) effect (Maeda et al. 2000; Matsumura 2007; Matsumura et al. 1987).

Liposomes are artificial phospholipid vesicles able to encapsulate water-soluble drugs in their aqueous inner space as well as lipophilic drugs in the phospholipid bilayer membrane. Since their accidental discovery by AlexBangham (Bangham et al. 1965a, b) in the early 1960s, liposomes have been described and classified in the literature as a colloidal drug delivery system. Caused by the recent rise in popularity of using the term “nano” in the pharmaceutical literature, liposomes are now increasingly being referred to as nanolipid vesicles, since they are actually the prototype of all nanodrug delivery vectors currently under development. The combined experience gained in over 40 years of liposome technology provides an irreplaceable source for new investigators in the emerging field of pharmaceutical and biomedical nanotechnology.

The target selectivity of the currently clinically approved lipid-based nanodrug delivery system, however, is neither cell specific nor specific for cell organelles such as mitochondria. The therapeutic efficiency of liposomes is mainly based on their inability to extravasate in healthy tissue, thus avoiding side effects such as cardiotoxicity in the case of doxorubicin. Following their EPR-driven accumulation at the solid tumor site, liposomes then slowly disintegrate releasing the free drug into the tumor interstitium. Depending on the drug’s physicochemical properties, it

eventually diffuses passively to some extent into the tumor cell interior, where it will either randomly be able to interact with its target (nuclear DNA in the case of doxorubicin) or become substrate of the p-glycoprotein resulting in its expulsion from the tumor cell.

To efficiently and selectively eradicate carcinoma cells, the cytotoxic drug not only needs to be delivered to the solid tumor and into the tumor cell, but also to the particular target *inside* the cell. Tumor-specific *subcellular* drug delivery constitutes a new approach for the chemotherapy of cancer. This approach becomes increasingly feasible as the particular mechanism of action, i.e., the molecular targets of anticancer drugs are being recognized. Transporting the cytotoxic drug to its intracellular target could also overcome MDR by bypassing the p-glycoprotein. The drug would literally be "hidden" from the p-glycoprotein inside the delivery system until it becomes selectively released at the particular intracellular site of action.

Several conventional anticancer drugs, such as the already mentioned doxorubicin or cisplatin, have no direct effect on mitochondria (Fulda et al. 1998). These conventional chemotherapeutic agents elicit mitochondrial permeabilization in an indirect fashion by induction of endogenous effectors that are involved in the physiologic control of apoptosis (Costantini et al. 2000a, b). However, a variety of clinically approved drugs such as paclitaxel (Andre et al. 2000, 2002; Ferlini et al. 2003; Kidd et al. 2002), VP-16 (etoposide) (Fujino et al. 2002; Itoh et al. 2003), vinorelbine (Andre et al. 2000) as well as an increasing number of experimental anticancer drugs such as betulinic acid, lonidamine, CD-437 (a synthetic retinoids), and ceramide (reviewed in Costantini et al. 2000a, b) have been found to act directly on mitochondria resulting in triggering apoptosis. These agents may induce apoptosis in circumstances in which conventional drugs fail to act because endogenous apoptosis-inducing pathways, e.g., such as those involving p53, death receptors, or apical caspase activation, are disrupted, leading to the apoptosis resistance of tumor cells. For example, several in vitro and in vivo studies have shown that the synthetic retinoid CD437 is able to induce apoptosis in human lung, breast, cervical and ovarian carcinoma cells (reviewed in Costantini et al. 2000a, b). It could be demonstrated that, in intact cells, CD437-dependent caspase activation is preceded by the release of cytochrome *c* from mitochondria (Marchetti et al. 1999). Moreover, it was shown that, when added to isolated mitochondria, CD437 causes membrane permeabilization and that this effect is prevented by inhibitors of the mPTPC such as cyclosporine A. CD437 constitutes an experimental drug, which exerts its cytotoxic effect via the mPTPC, i.e., by acting directly at the surface or inside of mitochondria.

The successful development of efficient anticancer drugs, whose cytotoxic effects depend on their direct interaction with mitochondria inside of living cells, will largely depend on understanding and controlling the intracellular distribution of these drugs after having been internalized by the cell. The problems linked to a drug's "intracellular bioavailability" will be the subject of the next section.

Cytosolic Barriers to Mitochondrial Drug Delivery

Due to the presence of a widespread mitochondrial network in almost all cells, the view that, once inside the cell, any drug molecule including oligonucleotides and plasmid DNA will eventually interact with components of the mitochondrial membrane and – depending on their physicochemical properties – perhaps diffuse into the matrix appears plausible. Certainly, statistical collision may indeed lead to an interaction of low-molecular-weight compounds or DNA with mitochondria. But so will the interaction of this molecule with other targets inside the cell causing a decrease of the subcellular (organelle-specific) bioavailability of the drug (Weissig et al. 2004).

While barriers to drug delivery between the site of administration and the target tissue are well defined and recognized, intracellular barriers preventing an even distribution of drug or DNA molecules throughout the cell, which the therapeutic agent has to overcome in order to reach its subcellular target site, are largely underappreciated and less well characterized. Factors significantly interfering with the free diffusion of solutes in the cytosol are firstly the fluid-phase viscosity of the cytoplasm, secondly collisional interactions due to macromolecular crowding, and thirdly binding to intracellular components (Lukacs et al. 2000; Seksek et al. 1997). The impact on cytoplasmic solute diffusion of the combination of these three factors is measurable and is expressed as the translational diffusion coefficient. For example, Lukacs et al. (2000) have used spot photobleaching to measure the translational diffusion of fluorescein-labeled double-stranded DNA fragments of different sizes after microinjection into the cytoplasm of HeLa cells. As one would expect, the diffusion rate decreased with increasing size of the DNA and was actually immeasurably slow for DNAs of 3kb or greater. Recently, it was shown (Dauty and Verkman 2005) that the size-dependent reduction in the mobility of large macromolecules or DNA fragments was abolished after the disruption of actin microfilaments, thereby suggesting this component of the cytoskeleton as the principal structure that limits passive diffusion through the cytoplasm. Using microinjection and electroporation approaches in the presence of drugs that alter the dynamics and organization of the cytoskeleton, Vaughan and Dean demonstrated that also microtubules seem to be involved in plasmid trafficking to the nucleus (Vaughan and Dean 2006). In addition, by coinjecting inhibitory antibodies, these authors found that dynein likely facilitates this movement. Since Vermerk's studies were focused on measuring rates of diffusion on small time scales (seconds to minutes), while Vaughan and Dean were assessing the movement of DNA over periods of hours, it has been concluded that the disruption of the actin network may play a significant role in local movement over very short times, but this role may be lessened over time, as microtubules play the predominant role in DNA movement (Vaughan and Dean 2006).

Physicochemical Properties Determining a Drug's Mitochondria-Specific Bioavailability

When considering the intracellular fate of a drug, i.e., its "subcellular biodistribution," besides the intracellular environment, the nature of the drug itself is equally important. The mode of interaction of the drug with intracellular components is actually determined by the "makeup" of the drug. Based on own experimental and on published data, Richard Horobin has developed a QSAR modeling approach for predicting the cellular uptake, the intracellular distribution, and the site of intracellular accumulation for fluorescent probes and dyes (Horobin 2001). He demonstrates that the amphiphatic character, the size of the aromatic system, the electric charge, the overall size, the presence of planar aromatic moieties, the lipophilicity, and the acid/base properties all determine the intracellular disposition of these molecules. Horobin's QSAR approach has most recently been applied to defining the physicochemical parameters required for mitochondrial targeting of low-molecular-weight compounds (Horobin et al. 2007). The mitochondrial targeting of almost 80% out of over 100 randomly chosen compounds known to be localized, or active, in mitochondria could be predicted using two sets of QSAR models, one specifying accumulation of lipophilic cations and the other of lipophilic weak acids (Horobin et al. 2007). The author's approach also allowed for uptake into intracellular sites besides mitochondria, such as endoplasmic reticulum (ER) and generalized biomembranes. Table 1 summarizes numerically expressed physicochemical criteria for entry into the cell and for mitochondrial uptake as well as for localization to other cell organelles.

Since this approach fails to predict the observed mitochondrial activity of about 20% of the selected compounds, the authors have explored an alternative predictive procedure, namely a first-principles physicochemical approach based on the Fick–Nernst–Planck equations (Trapp and Horobin 2005). The details of this approach are, however, beyond the scope of this chapter.

Table 1 Quantitative physicochemical molecular parameter ("QSAR decision rules") allowing the prediction of the intracellular distribution of low-molecular compounds. Reprinted with permission from (Weissig et al., 2007a)

Event	Physicochemical criteria
Entry into the cell	$8 > AI$ or $\log P > 0$; $CBN < 40$
Selective mitochondrial uptake of cations	$5 > \log P_{cation} > 0$
Selective mitochondrial uptake of cations according to "First-principles model" (see text)	$5 > \log P > -2$
Selective mitochondrial uptake of acids	$pK_a = 7 \pm 3$; $5 > \log P_{less\ ionized\ species} > 0$
Non-selective cation uptake in ER & membranes	$AI > 3.5$; $8 > \log P_{cation} > 5$
Non-selective uptake in membranes	$AI > 3.5$; $8 > \log P_{less\ ionized\ species} > 5$

AI = Amphipathic index; CBN = Conjugated bond number; Log P = log of the octanol water partition coefficient—for details see references in (Horobin et al., 2007)

Mitochondria-Targeted Nanodrug Delivery Systems

Utilizing a cell-based assay for screening a chemical library containing about 16,000 compounds, a small molecule called F16 was recently identified as a potent anticancer agent (Fantin et al. 2002). This molecule was found to accumulate almost completely in the mitochondrial matrix in response to the elevated mitochondrial membrane potential. Concentration of F16 in the matrix leads to depolarization, opening of the permeability transition pore, cytochrome *c* release, cell cycle arrest, and consequently cell death (Fantin et al. 2002). This novel mitochondriotropic agent F16 was found to selectively inhibit proliferation of mammary epithelial, *neu*-overexpressing cells, as well as a variety of mouse mammary tumor and human breast cancer cell lines. It appears remarkable that F16 was selected out of 16,000 small molecules for its specific inhibition of cell proliferation without any presumption of a mitochondrial target (Hockenbery 2002). Obviously, with F16 a compound was found, which fulfills Horobin's QSAR criteria for mitochondrial accumulation and displays mitochondria-based cytotoxicity. Without a doubt, screening large chemical libraries will keep revealing potent drug candidates, and as it is in the case of F16, also drug candidates able to accumulate at their intracellular site of action. Unfortunately, even compounds such as F16 still need to be delivered selectively to the diseased tissue, i.e., to the tumor. Countless extremely potent drug candidates identified in vitro have been dropped from further development due to their poor solubility in aqueous media, which drastically reduces their bioavailability. On the other hand, quite soluble compounds have limited ability to cross lipid membranes, making the cell interior almost inaccessible to them. The general question seems to be whether it is easier to design a drug, which combines all essential physicochemical properties for providing high systemic, tissue, cellular, and subcellular bioavailability with the desired high pharmacological activity or to design a tissue-, cell-, and organelle-specific delivery technology applicable to a wide variety of small pharmacologically active molecules. With the development of liposome-based drug delivery systems, a first important step has been taken. Liposomes provide a nanotechnological drug delivery platform, which is equally applicable to soluble and insoluble drug candidates. Liposomes are able to selectively deliver cancer drugs to the site of a solid tumor while largely avoiding healthy tissue, but they do not provide any subcellular (i.e., mitochondrial) targeting capability.

An accidental discovery in the late 1990s has shown a principal way toward the design of subcellular, i.e., mitochondria-specific nanodrug delivery systems. As it will be described in more detail in the next section, a known mitochondriotropic molecule, dequalinium chloride, was found under certain experimental conditions to be able to self-assemble into stable nanovesicular liposome-like structures, called DQAsomes. Although Horobin's QSAR criteria (Horobin et al. 2007) have been established for small molecules only, apparently the self-assembly of one of such molecules gives rise to nanostructures with likewise mitochondriotropic properties.

DQAsomes as the Prototype for Mitochondria-Specific Nanotechnology

Symmetric amphiphilic molecules, in which two hydrophilic residues are linked by a hydrophobic segment, are generally known as "bola-lipids" based on their resemblance to an old South American hunting weapon. Well-characterized bola-amphiphiles are archaebacterial lipids, which usually consist of two glycerol backbones connected by two hydrophobic chains. The self-assembly behavior of such bipolar archaeal lipids has been extensively studied and it has been shown that they can self-associate into mechanically very stable monolayer membranes (Gambacorta et al. 1995).

During the middle of the 1990s, while screening mitochondriotropic drugs potentially able to interfere with the mitochondrial DNA metabolism (Rowe et al. 2001) in *P. falciparum*, it was discovered (by accident) that dequalinium chloride (Fig. 1a), a bola-amphiphilic drug, tends to self-associate into colloidal structures when sonicated as an aqueous suspension. Employing transmission electron

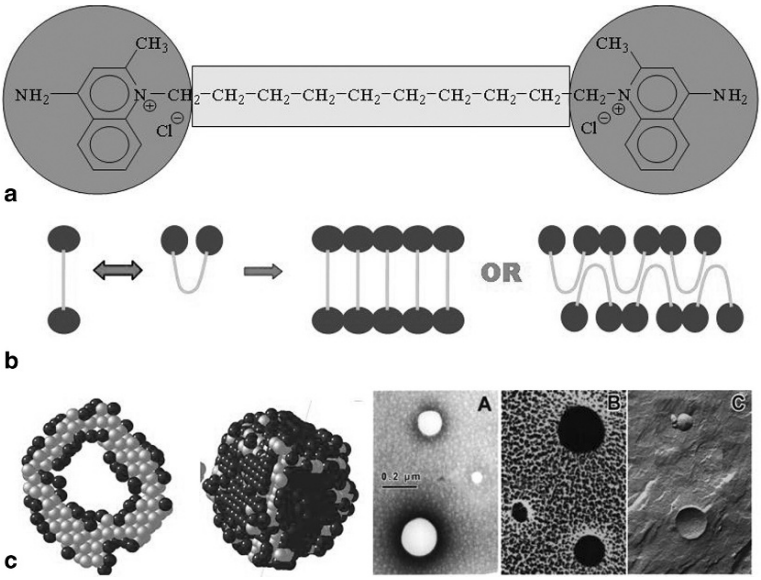

Fig. 1 The self-assembly behavior of dequalinium chloride leads to the formation of liposome-like vesicles called "DQAsomes". (**a**) Chemical structure of dequalinium chloride, with gray areas indicating the hydrophilic and the hydrophobic part of the molecule, respectively. (**b**) Theoretical possible conformations of dequalinium chloride, i.e., stretched vs. horseshoe conformation, leading to either a monolayer or a bilayer membranous structure following the process of self-assembly. (**c**) *Left panel*: Monte Carlo computer simulations demonstrate the possible self-assembly of dequalinium chloride into vesicles. (**c**) *Right panel*: Electron microscopic images of vesicles ("DQAsomes") made from dequalinium chloride, from left to right: negatively stained, rotary shadowed, freeze fractured. Whole figure reprinted with permission from Cheng et al. (2007; #11419)

microscopy (Fig. 1c, right panel) as well as photon correlation spectroscopy, it was confirmed that dequalinium forms upon probe sonication spherical aggregates with a diameter between about 70 and 700 nm. Freeze fracture electron microscopic images (Fig. 1c, right panel, c) showed both convex and concave fracture faces, thereby demonstrating the liposome-like aggregation of dequalinium. At the time of their accidental discovery, these vesicles were termed DQAsomes (i.e., dequalinium-based liposome-like vesicles; pronounced *dequasomes*) (Weissig et al. 1998).

A structural difference between dequalinium and archaeal lipids, however, lies in the number of bridging hydrophobic chains between the polar head groups. In contrast to common arachaeal lipids, dequalinium has only one alkyl chain that connects the two cationic hydrophilic head groups. Therefore, theoretically two different conformations within a self-assembled layer structure are imaginable. While the stretched conformation would give rise to the formation of a monolayer, assuming the horseshoe conformation would result in the formation of a bilayer (Fig. 1b).

In a series of papers (D'Souza et al. 2003, 2005; Lasch et al. 1999; Weissig et al. 2000, 2001), DQAsomes have been established as the first mitochondria-targeted transfection vector making mitochondrial gene therapy feasible (D'Souza et al. 2007). Of more interest in the context of this book, however, is that DQAsomes have also been explored as a mitochondria-targeted carrier system for small drug molecules, in particular anticancer drugs known to trigger apoptosis via direct action on mitochondria. For example, paclitaxel-loaded DQAsomes (Fig. 2) have been tested for their ability to inhibit the growth of human colon cancer tumors in nude mice (Cheng et al. 2005) and the data suggest that encapsulation of paclitaxel in DQAsomes leads to improved efficacy.

To test whether the increased efficacy observed in vivo correlates with an increased apoptotic activity of encapsulated paclitaxel drug over the free drug, human colon cancer cells were incubated with either the free drug, with empty

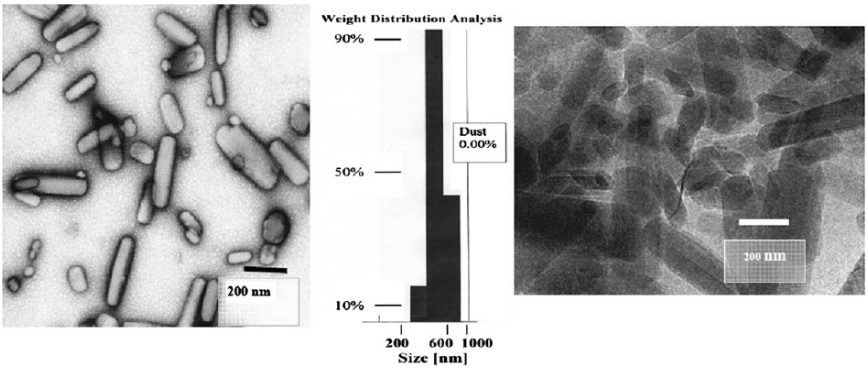

Fig. 2 Paclitaxel encapsulated into DQAsomes. *Left panel*: Transmission electron microscopic image (uranyl acetate staining); *Middle panel*: Size distribution; *Right panel*: Cryo-electron microscopic image. Reprinted with permission from Cheng et al. (2005; #11105)

DQAsomes, with a mixture of empty DQAsomes and the free drug and with the DQAsomal encapsulated drug. Following staining of the treated cells with the DNA-binding fluorophore Hoechst 33258, apoptotic nuclei showing the typical apoptotic condensation and fragmentation of chromatin were counted and expressed as percent of the total number of nuclei. The left panel in Fig. 3 shows that, under identical incubation conditions, 10 nM paclitaxel encapsulated in DQAsomes more than double the number of apoptotic nuclei in comparison to the control, in which cells were treated with a mixture of empty DQAsomes and 10 nM free paclitaxel.

Likewise, a DNA ladder caused by DNA fragmentation typical for apoptosis could be detected upon incubation of colon cancer cells with 10 nM DQAsomal encapsulated paclitaxel, but not upon incubation with the free drug either alone or in mixture with empty DQAsomes (Fig. 3, right panel).

Incubating the cells for the same period of time, the amount of free paclitaxel had to be increased at least fivefold over the amount of DQAsomal encapsulated drug in order to generate a DNA ladder (not shown).

Considering that paclitaxel, generally known as an antimicrotubule agent, has also been demonstrated to trigger apoptosis by directly acting on mitochondria (Andre et al. 2000, 2002; Carre et al. 2002; Kidd et al. 2002), it can be inferred that encapsulating paclitaxel into a mitochondria-specific nanodrug delivery systems appears to increase the subcellular, i.e., mitochondrial, bioavailability of the drug.

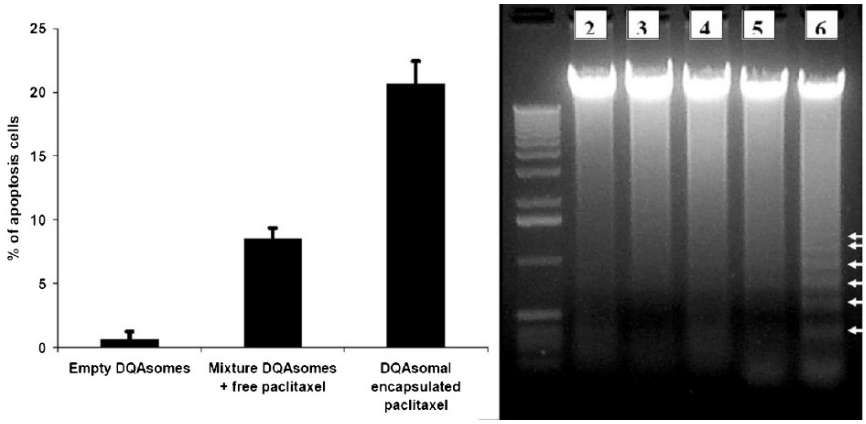

Fig. 3 DQAsomal encapsulated paclitaxel triggers apoptosis in vitro. *Left panel*: Human colo 25 colon cancer cells were treated in triplicates with empty DQAsomes (20 nM DQA), with a mixture of empty DQAsomes (20 nM) and free paclitaxel (10 nM) and DQAsomal encapsulated paclitaxel (20 nM DQA/10 nM paclitaxel). In each case, approximately 400 cells were counted. *Right panel*: Human colo 25 colon cancer cells were incubated for 30 h with buffer (lane 2), 20 nM empty DQAsomes (lane 3), 10 nM free paclitaxel (lane 4), a mixture of 20 nM empty DQAsomes and 10 nM free paclitaxel (lane 5) and 20 nM DQAsomes with 10 nM encapsulated paclitaxel (lane 6). *White arrow heads* indicate the apoptotic DNA ladder D'Souza et al. 2007a

Mitochondria-Specific Nanolipid Vesicles (Liposomes)

It has been well established that the surface modification of liposomes, i.e., the attachment of small and large molecules (e.g., antibodies, polyethylene glycol, carbohydrates, peptides) significantly alters the vesicle's behavior in biological milieu. The general procedure for modifying the surface of liposomes involves their preparation in the presence of hydrophilic molecules, which have been artificially hydrophobized via linkage to fatty acid or phospholipid derivatives. As a result, the hydrophilic moiety becomes literally "anchored" to the liposomal surface (Niedermann et al. 1991; Torchilin et al. 2003; Weissig and Gregoriadis 1993; Weissig et al. 1986, 1989). This established method was used to render liposomes mitochondria specific, as schematically shown in Fig. 4.

Mitochondriotropic triphenylphosphonium cations were hydrophobized by reacting triphenylphosphine with stearylbromide. The resulting stearyl-triphenylphosphonium bromide (STPP) was then added to the solution of phospholipids in chloroform used to prepare liposomes. STPP liposomes, i.e., liposomes with surface-linked triphenylphosphonium cations, were isolated using a Sepharose CL-4B column and characterized by ^{31}P-NMR, size distribution analysis, and zeta potential measurements (Boddapati et al. 2005).

Figure 5 shows a confocal fluorescence microscopic image of breast cancer cells that have been incubated for 2h with fluorescence-labeled STPP liposomes. Panels a and b show mitochondria in red and nuclei in blue, respectively. Panel c depicts STPP liposomes labeled with a green fluorophore. In panel d, the red, blue and

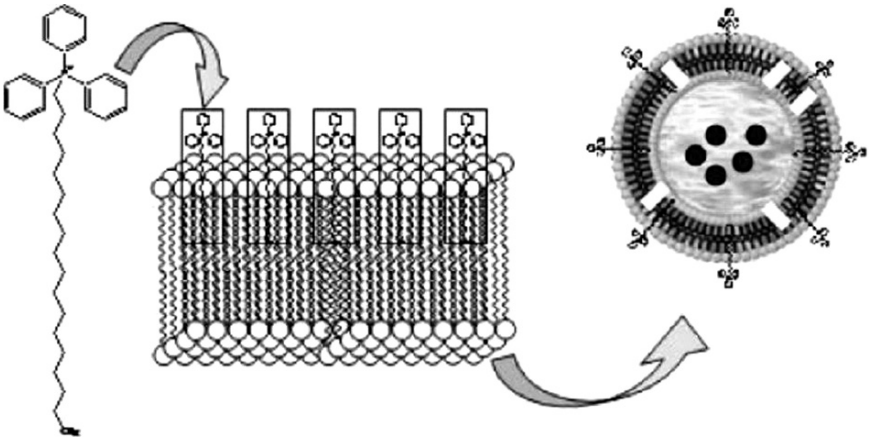

Fig. 4 Mitochondriotropic nanolipid vesicles (liposomes). Liposomes are rendered mitochondria-specific via surface modification with hydrophobized mitochondriotropic residues. Stearyl-triphenylphosphonium bromide (STPP) was synthesized from triphenylphosphine and stearylbromide. Liposomes were then prepared in the presence of STPP. Triphenylphosphonium cations anchored in the inner monolayer are not shown. *Filled black circles* represent liposomal encapsulated water-soluble drug molecules, *gray rectangles* represent lipid-soluble drugs embedded in the liposomal phospholipid bilayer membrane (schematic presentation, not to scale)

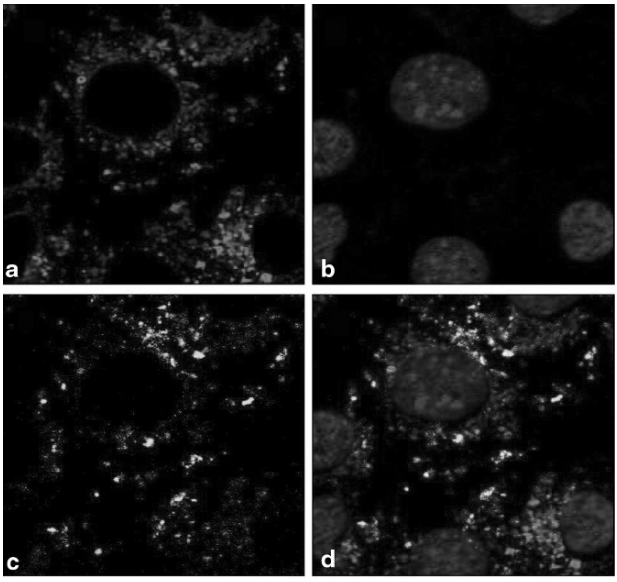

Fig. 5 Confocal fluorescence microscopic image of cells incubated with mitochondriotropic liposomes. 4T1 breast cancer cells were incubated for 2 h with 10 mol% STPP liposomes. (**a**) Mitochondria stained with Mitotracker, (**b**) Cell nuclei stained with Hoechst 33342; (**c**) STPP liposomes labeled with NBD-PE; (**d**) Overlaid images A, B, and C (Boddapati et al. 2007, unpublished)

green images have been overlaid. It can clearly be seen that almost all green dots either colocalize with mitochondria (depicted in yellow) or are extremely close to mitochondria. Based on the fact that the green fluorophore, 7-nitrobenz-2-oxa-1,3-diazol, in STPP liposomes was covalently linked to phosphatidylethanolamine and not to the mitochondriotropic entity, i.e., to STPP, it can be concluded from Fig. 5 that at least partially intact phospholipid vesicles must have accumulated at the site of mitochondria.

The exploration of mitochondriotropic liposomes for the delivery of hydrophobic proapoptotic drugs known to act at the site of mitochondria is currently in progress in the author's laboratory.

Gold Nanoparticles Suited for Mitochondrial Targeting

Solid nanoparticles (in distinction to "hollow" vesicular DQAsomes and liposomes described above) offer new and unique applications for diagnostic and therapy of human diseases. New nanodiagnostic tools, including quantum dots and gold nanoparticles (AuNPs), promise increased sensitivity, multiplexing capabilities, and reduced costs for many diagnostic applications (Azzazy et al. 2006). A particular promising application lies in the area of anticancer therapy. For example, AuNPs

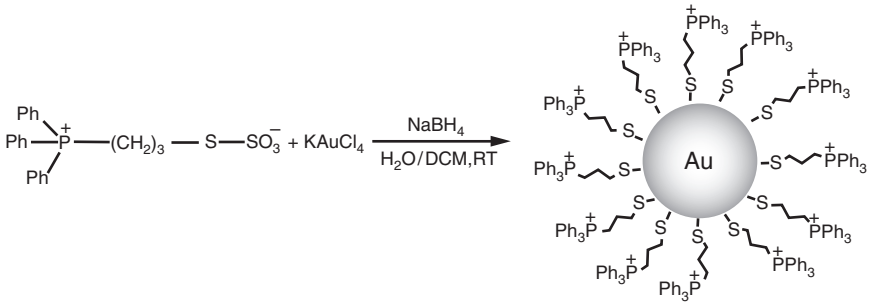

Fig. 6 Gold nanoparticles bearing surface-linked mitochondriotropic ligands. Reaction scheme for the surface modification of gold nanoparticles with triphenylphosphonium cations. The image was graciously provided by Neil Bricklebank and reprinted in slightly modified form from Ju-Nam et al. (2006); Copyright permission was granted by Organic and Biomolecular Chemistry (The Royal Society of Chemistry)

can mediate hyperthermia induction and kill tumor cells upon laser irradiation, thereby functioning as a "thermal scalpel" (Everts 2007).

Attempts to introduce solid nanoparticles into mitochondrial medicine have been very limited so far. A very first step toward the utilization of solid nanoparticles for possible probing and manipulating mitochondrial functions was recently undertaken by coating the surface of AuNPs with mitochondriotropic triphenylphosphonium cations (Fig. 6). Ju-Nam et al. (2006) incubated potassium tetrachloroaurate with triphenyl-phosphonioalkylthiosulfate, a member of a whole group of newly synthesized zwitterions and added dropwise an aqueous solution of sodium borohydride.

After vigorous stirring for 24h, the authors were able to isolate 5–10 nm sized AuNPs with surface-attached triphenylphosphonium residues. Unfortunately, at the time of writing this chapter (August 2007), data regarding the intracellular distribution of such triphenylphosphonium cation bearing AuNPs were not yet available. Corresponding experiments are ongoing in the author's laboratory.

Summary

As a summary, Fig. 7 provides a schematic overview about mitochondria-specific nanotechnology currently under development. The mitochondria specificity of DQAsome is based upon the self-assembly of mitochondriotropic quinolinium-derived bola amphiphiles. Liposomes and AuNPs are being rendered mitochondria-specific via surface modification with mitochondriotropic low-molecular-weight compounds (Horobin et al. 2007). Quantum dots, the discussion of which was beyond the scope of this chapter, display mitochondria-specific antibodies on their surface (Azzazy et al. 2006; Medda et al. 2006; Zorov et al. 2004).

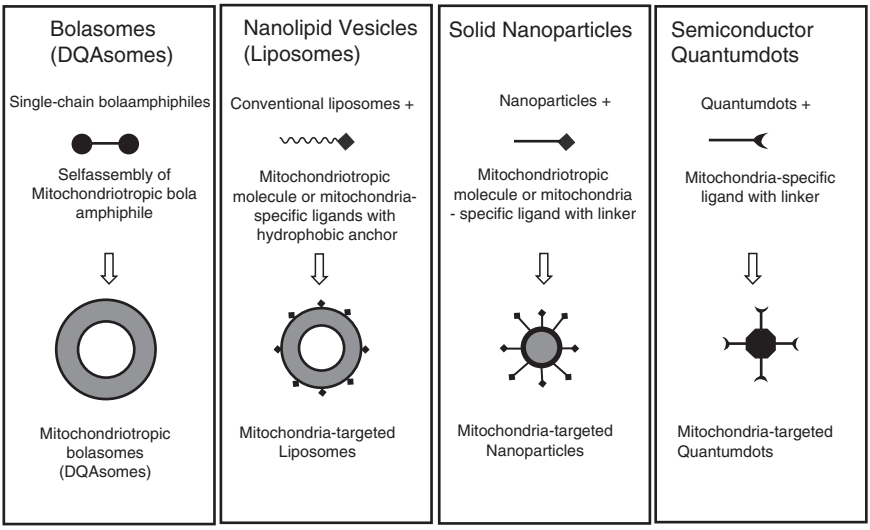

Fig. 7 Mitochondrial Nanotechnology (schematic overview). Reprinted with permission from Weissig et al. (2007b)

Each of the shown technologies opens unique approaches toward treatment and diagnostic of cancer. The incursion of nanotechnology into mitochondrial medicine is therefore expected to have a significant impact on oncology in the near future.

References

Andre N, Braguer D, Brasseur G, Goncalves A, Lemesle-Meunier D, et al. 2000. Paclitaxel induces release of cytochrome c from mitochondria isolated from human neuroblastoma cells. *Cancer Res* 60: 5349–53

Andre N, Carre M, Brasseur G, Pourroy B, Kovacic H, et al. 2002. Paclitaxel targets mitochondria upstream of caspase activation in intact human neuroblastoma cells. *FEBS Lett* 532: 256–60

Azzazy HM, Mansour MM, Kazmierczak SC. 2006. Nanodiagnostics: a new frontier for clinical laboratory medicine. *Clin Chem* 52: 1238–46

Bangham AD, Standish MM, Watkins JC. 1965a. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J Mol Biol* 13: 238–52

Bangham AD, Standish MM, Weissmann G. 1965b. The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. *J Mol Biol* 13: 253–9

Boddapati SV, Tongcharoensirikul P, Hanson RN, D'Souza GG, Torchilin VP, Weissig V. 2005. Mitochondriotropic liposomes. *J Liposome Res* 15: 49–58

Carre M, Carles G, Andre N, Douillard S, Ciccolini J, et al. 2002. Involvement of microtubules and mitochondria in the antagonism of arsenic trioxide on paclitaxel-induced apoptosis. *Biochem Pharmacol* 63: 1831–42

Cheng SM, Pabba S, Torchilin VP, Fowle W, Kimpfler A, Schubert R, Weissig V. 2005. Towards mitochondria-specific delivery of apoptosis-inducing agents: DQAsomal incorporated paclitaxel. *J Drug Deliv Sci Technol* 15: 81–6

Cheng SM, Boddapati SV, D'Souza GM, Weissig V. 2007. DQAsomes as Mitochondria-Targeted Nano-Carriers for Anticancer Drugs. In *Nanotechnology for Cancer Therapeutics*, ed. M Amiji, pp. 787-802. FL, USA/PA, USA: CRC/Taylor & Francis Group LLC

Costantini P, Jacotot E, Decaudin D, Kroemer G. 2000a. Mitochondrion as a novel target of anticancer chemotherapy. *J Natl Cancer Inst* 92: 1042–53

Costantini P, Jacotot E, Decaudin D, Kroemer G. 2000b. Mitochondrion as a novel target of anticancer chemotherapy. *J Natl Cancer Inst* 92: 1042–53

Dauty E, Verkman AS. 2005. Actin cytoskeleton as the principal determinant of size-dependent DNA mobility in cytoplasm: a new barrier for non–viral gene delivery. *J Biol Chem* 280: 7823–8

Dias N, Bailly C. 2005. Drugs targeting mitochondrial functions to control tumor cell growth. *Biochem Pharmacol* 70: 1–12

D'Souza GG, Rammohan R, Cheng SM, Torchilin VP, Weissig V. 2003. DQAsome-mediated delivery of plasmid DNA toward mitochondria in living cells. *J Control Release* 92: 189–97

D'Souza GG, Boddapati SV, Weissig V. 2005. Mitochondrial leader sequence-plasmid DNA conjugates delivered into mammalian cells by DQAsomes co-localize with mitochondria. *Mitochondrion* 5: 352–8

D'Souza GG, Boddapati SV, Weissig V. 2007. Gene therapy of the other genome: the challenges of treating mitochondrial DNA defects. *Pharm Res* 24: 228–38

D'Souza GG, Cheng SM, Boddapati SV, Horobin RW, Weissig V. 2007a. Nanocarrier-assited sub-cellular targeting to the site of mitochondria improves the pro-apoptotic activity of paclitaxel. J Drug Targeting 16(7): 578–85

Everts M. 2007. Thermal scalpel to target cancer. *Expert Rev Med Devices* 4: 131–6

Fantin VR, Berardi MJ, Scorrano L, Korsmeyer SJ, Leder P. 2002. A novel mitochondriotoxic small molecule that selectively inhibits tumor cell growth. *Cancer Cell* 2: 29–42

Ferlini C, Raspaglio G, Mozzetti S, Distefano M, Filippetti F, et al. 2003. Bcl-2 Down-Regulation Is a Novel Mechanism of Paclitaxel Resistance. *Mol Pharmacol* 64: 51–8

Fujino M, Li XK, Kitazawa Y, Guo L, Kawasaki M, et al. 2002. Distinct pathways of apoptosis triggered by FTY720, etoposide, and anti-Fas antibody in human T-lymphoma cell line (Jurkat cells). *J Pharmacol Exp Ther* 300: 939–45

Fulda S, Susin SA, Kroemer G, Debatin KM. 1998. Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells. *Cancer Res* 58: 4453–60

Gambacorta A, Gliozi A, De Rosa M. 1995. Archaeal lipids and their biotechnological applications. *World J Microbiol Biotechnol* 11: 115–31

Hockenbery DM. 2002. A mitochondrial Achilles' heel in cancer? *Cancer Cell* 2: 1–2

Horobin RW. 2001. Uptake, distribution and accumulation of dyes and fluorescent probes within living cells: a structure–activity modelling approach. *Adv Colour Sci Technol* 4: 101–7

Horobin RW, Trapp S, Weissig V. 2007. Mitochondriotropics: a review of their mode of action, and their application fro drug and DNA delivery to mammalian mitochondria. *J Control Release* 121: 125–136

Itoh M, Noutomi T, Toyota H, Mizuguchi J. 2003. Etoposide-mediated sensitization of squamous cell carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced loss in mitochondrial membrane potential. *Oral Oncol* 39: 269–76

Ju-Nam Y, Bricklebank N, Allen DW, Gardiner PH, Light ME, Hursthouse MB. 2006. Phosphonioalkylthiosulfate zwitterions - new masked thiol ligands for the formation of cationic functionalised gold nanoparticles. *Org Biomol Chem* 4: 4345–51

Kidd JF, Pilkington MF, Schell MJ, Fogarty KE, Skepper JN, et al. 2002. Paclitaxel affects cytosolic calcium signals by opening the mitochondrial permeability transition pore. *J Biol Chem* 277: 6504–10

Lasch J, Meye A, Taubert H, Koelsch R, Mansa-ard J, Weissig V. 1999. Dequalinium vesicles form stable complexes with plasmid DNA which are protected from DNase attack. *Biol Chem* 380: 647–52

Lukacs GL, Haggie P, Seksek O, Lechardeur D, Freedman N, Verkman AS. 2000. Size-dependent DNA mobility in cytoplasm and nucleus. *J Biol Chem* 275: 1625–9

Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release* 65: 271–84

Marchetti P, Zamzami N, Joseph B, Schraen-Maschke S, Mereau-Richard C, et al. 1999. The novel retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphtalene carboxylic acid can trigger apoptosis through a mitochondrial pathway independent of the nucleus. *Cancer Res* 59: 6257–66

Matsumura Y. 2007. Preclinical and clinical studies of anticancer drug-incorporated polymeric micelles. *J Drug Target* 15: 507–17

Matsumura Y, Oda T, Maeda H. 1987. [General mechanism of intratumor accumulation of macromolecules: advantage of macromolecular therapeutics]. *Gan To Kagaku Ryoho* 14: 821–9

Medda R, Jakobs S, Hell SW, Bewersdorf J. 2006. 4Pi microscopy of quantum dot-labeled cellular structures. *J Struct Biol* 156: 517–23

Niedermann G, Weissig V, Sternberg B, Lasch J. 1991. Carboxyacyl derivatives of cardiolipin as four-tailed hydrophobic anchors for the covalent coupling of hydrophilic proteins to liposomes. *Biochim Biophys Acta* 1070: 401–8

Rowe TC, Weissig V, Lawrence JW. 2001. Mitochondrial DNA metabolism targeting drugs. *Adv Drug Deliv Rev* 49: 175–87

Seksek O, Biwersi J, Verkman AS. 1997. Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus. *J Cell Biol* 138: 131–42

Torchilin VP, Weissig V, Martin FJ, Heath TD, New RRC. 2003. Surface Modification of Liposomes. In *Liposomes - A Practical Approach*, ed. VP Torchilin, Weissig, V, pp. 193–230. Oxford: Oxford University press

Trapp S, Horobin RW. 2005. A predictive model for the selective accumulation of chemicals in tumor cells. *Eur Biophys J* 34: 959–66

Vaughan EE, Dean DA. 2006. Intracellular trafficking of plasmids during transfection is mediated by microtubules. *Mol Ther* 13: 422–8

Weissig V, Gregoriadis G. 1993. Coupling of Aminogroup-Bearing Ligands to Liposomes. In *Liposome Technology*, ed. G Gregoriadis, pp. 231–48. Boca Raton, Ann Arbor, London, Tokyo: CRC

Weissig V, Lasch J, Klibanov AL, Torchilin VP. 1986. A new hydrophobic anchor for the attachment of proteins to liposomal membranes. *FEBS Lett* 202: 86–90

Weissig V, Lasch J, Gregoriadis G. 1989. Covalent coupling of sugars to liposomes. *Biochim Biophys Acta* 1003: 54–7

Weissig V, Lasch J, Erdos G, Meyer HW, Rowe TC, Hughes J. 1998. DQAsomes: a novel potential drug and gene delivery system made from Dequalinium. *Pharm Res* 15: 334–7

Weissig V, Lizano C, Torchilin VP. 2000. Selective DNA release from DQAsome/DNA complexes at mitochondria-like membranes. *Drug Deliv* 7: 1–5

Weissig V, D'Souza GG, Torchilin VP. 2001. DQAsome/DNA complexes release DNA upon contact with isolated mouse liver mitochondria. *J Control Release* 75: 401–8

Weissig V, Cheng S-M, D'Souza G. 2004. Mitochondrial Pharmaceutics. *Mitochondrion* 3: 229–44

Weissig V, Boddapati SV, D'Souza GGM, Horobin RH. 2007a. Functionalization of Pharmaceutical Nanocarriers for Mitochondria-targeted Drug and DNA Delivery. In *Multifunctional Pharmaceutical Nanocarriers*, ed. VP Torchilin: Springer: Berlin

Weissig V, Boddapati SV, Jabre L, D'Souza GGM. 2007b. Mitochondria-specific nanotechnology. *Nanomedicine* 2: 275–85

Zorov DB, Kobrinsky E, Juhaszova M, Sollott SJ. 2004. Examining intracellular organelle function using fluorescent probes: from animalcules to quantum dots. *Circ Res* 95: 239–52

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