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Curbing Cancer's Sweet Tooth: Is There a Role for MnSOD in Regulation of the Warburg Effect?

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Abstract

Reactive oxygen species (ROS), while vital for normal cellular function, can have harmful effects on cells, leading to the development of diseases such as cancer. The Warburg effect, the shift from oxidative phosphorylation to glycolysis, even in the presence of adequate oxygen, is an important metabolic change that confers many growth and survival advantages to cancer cells. Reactive oxygen species are important regulators of the Warburg effect. The mitochondria-localized antioxidant enzyme manganese superoxide dismutase (MnSOD) is vital to survival in our oxygen-rich atmosphere because it scavenges mitochondrial ROS. MnSOD is important in cancer development and progression. However, the significance of MnSOD in the regulation of the Warburg effect is just now being revealed, and it may significantly impact the treatment of cancer in the future.

Keywords

mitochondria; manganese superoxide dismutase; Warburg effect; cancer; reactive oxygen species

1. Introduction

Reactive oxygen species (ROS) are produced as a result of oxygen metabolism (Fridovich, 1978). While ROS can have harmful effects on different cellular components (lipids, proteins, and DNA), ROS are also important mediators of myriad cellular processes, such as cell growth and differentiation (Boonstra and Post, 2004), adhesion, apoptosis, and the immune response (Droge, 2002); and they can participate as second messengers in cellular signaling (Forman et al., 2004; Gough and Cotter, 2011; Rhee et al., 2003; Valko et al., 2007). A precise ratio of ROS production to destruction exists in the cell, and disruption of this balance causes abnormal ROS signaling, contributing to disease development that includes neurological disorders (Waris and Ahsan, 2006) and cancer (Gius and Spitz, 2006; Valko et al., 2007; Waris and Ahsan, 2006).

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Under physiological conditions, mitochondria are the major sites of ROS production in the cell, and the superoxide radical ($O_2^{\bullet -}$) is the primary ROS generated from respiration by this organelle (Adam-Vizi and Chinopoulos, 2006; Hoye et al., 2008). Superoxide radicals contribute to the generation of other ROS, such as reactive nitrogen species (RNS) (Huie and Padmaja, 1993). ROS affect cellular function by altering the activities of proteins, including protein tyrosine and serine/threonine phosphatases (Wright et al., 2009), mitogen-activated (Schafer et al., 2003; Wang et al., 1998) and serine/threonine protein kinases (Poli et al., 2004), as well as myriad transcription factors, such as AP-1 (Abate et al., 1990), HIF-1 (Galanis et al., 2008), p53 (Fojta et al., 1999; Hainaut and Milner, 1993; Sun et al., 2003), and NF-κB (Kabe et al., 2005).

While ROS are vital for many cellular functions, altered basal levels of ROS can have striking effects on cellular homeostasis, leading to the development of a multitude of diseases. Aberrant ROS concentrations can occur through increased production of endogenous ROS, exogenous ROS-generating agents, and/or reduced ROS-scavenging capability. Manganese superoxide dismutase (MnSOD) is the major antioxidant enzyme of the cell because it is located in the mitochondria. Changes in MnSOD enzymatic function or protein expression can have serious repercussions on mitochondrial activity, resulting in changes in cellular function and, ultimately, the development of an assortment of illnesses (Miao and St. Clair, 2009; Oberley and Buettner, 1979).

The Warburg effect, the metabolic switch from oxidative phosphorylation to aerobic glycolysis, is a major hallmark of cancer (Warburg, 1956). In fact, overexpression of one or more glycolytic enzymes was observed in 70% of cancers worldwide representing 24 classes of neoplasia (Altenberg and Greulich, 2004). The need of cancer cells for glycolysis has made this pathway an attractive target for cancer therapy (Lopez-Lazaro, 2008). Changes in mitochondrial function are associated with the switch to glycolysis, but the role of MnSOD in initiation and maintenance of the Warburg effect is not well-established. In this review, we will discuss mitochondrial sources of ROS and the role of MnSOD in scavenging these ROS, as well as the importance of MnSOD in cancer development and progression. We will also discuss the Warburg effect, the part that ROS may play in controlling the Warburg effect, and the potential for MnSOD to regulate the metabolic switch to aerobic glycolysis.

2. Mitochondria and ROS Production

2.1. Mitochondria are a Major Source of ROS in the Cell

Mitochondria are the major sources of basal ROS (especially superoxide radicals) in the cell because of their role in oxygen metabolism (Halliwell and Gutteridge, 2007; Lenaz, 2001; Murphy, 2009). Various enzymes in the electron transport chain, in particular complex I (NADH-ubiquinone oxidoreductase) (Grivennikova and Vinogradov, 2006; Takeshige and Minakami, 1979) and complex III (ubiquinol-cytochrome *c* oxidoreductase) (Trumpower, 1990), are chief sites of superoxide generation (Brand, 2010). The site of superoxide production in complex I was identified as the region between the ferricyanide and ubiquinone reduction sites (Herrero and Barja, 2000) and localized to the iron-sulfur (FeS) centers N1a (Kushnareva et al., 2002) and N2 (Genova et al., 2001). Another source of superoxide production in complex I is its proton-pumping activity (Dlaskova et al., 2008).

Complex III produces superoxide using the ubisemiquinone intermediate of the Q-cycle (Trumpower, 1990) and releases superoxide on both sides of the inner membrane of mitochondria (Muller et al., 2004) into the intermembrane space (Han et al., 2001) and matrix (Chen et al., 2003). Complex II also contributes to mitochondrial superoxide production. The superoxide-producing site in complex II was suggested to be distal to the site of succinate oxidation (McLennan and Degli Esposti, 2000), and was narrowed down to being either the ubisemiquinone of the Q_0 site of the cytochrome bc_1 complex or the reduced cytochrome b_{566} (Zhang et al., 1998).

Other mitochondrial enzymes with no direct ties to the electron transport chain also produce ROS. Dihydroorotic dehydrogenase (important for pyrimidine synthesis) produces superoxide as a byproduct of the conversion of dihydroorotate to orotate (Forman and Kennedy, 1975; Forman and Kennedy, 1976). α-ketoglutarate dehydrogenase (α-KGDH), a tricarboxylic acid cycle (TCA cycle) enzyme, produces ROS in a NADH/NAD^+ ratio-dependent manner (Tretter and Adam-Vizi, 2004). The major site of ROS production in α-KGDH is the dihydrolipoyl dehydrogenase component (Starkov et al., 2004). Cytochrome P450s (Hanukoglu, 2006; Hanukoglu et al., 1993) and glycerophosphate dehydrogenase (Drahota et al., 2002; Miwa et al., 2003) are also important contributors to mitochondrial ROS production.

Superoxide can be considered a founder ROS, because it contributes to the production of other ROS that can cause cellular damage. Mitochondria have myriad FeS center containing proteins that are vulnerable to superoxide attack, leading to the release of free iron cations into mitochondria. These free iron cations participate in hydrogen peroxide-derived hydroxyl radical production through the Haber-Weiss reaction (Brookes et al., 2004; Fong et al., 1976; Raha and Robinson, 2000, 2001). Superoxide also reacts with nitric oxide to generate the reactive nitrogen species (RNS) peroxynitrite (Squadrito and Pryor, 1995), which can modify different amino acids in proteins, such as nitration of tyrosine (Abello et al., 2009) and oxidation of sulfhydryl groups (Radi et al., 1991a). Mitochondrial targets of peroxynitrite (Radi et al., 2002) include aconitase (Castro et al., 1994; Hausladen and Fridovich, 1994), glutathione peroxidase (Padmaja et al., 1998), complex I (Cassina and Radi, 1996; Murray et al., 2003; Radi et al., 1994; Riobo et al., 2001), complex II (Cassina and Radi, 1996; Radi et al., 1994), complex V (Radi et al., 1994), MnSOD (MacMillan-Crow et al., 1996; MacMillan-Crow et al., 1998; Yamakura et al., 1998), and Polγ (Bakthavatchalu et al., 2011).

2.2. Ways to Scavenge Mitochondrial ROS

Several enzyme systems exist in cells to combat the deleterious effects of ROS (Andreyev et al., 2005; Koehler et al., 2006). Superoxide dismutases (SODs) are the major superoxide-detoxifying enzymes of the cell (Fridovich, 1989). SODs catalyze the dismutation of superoxide to molecular oxygen and hydrogen peroxide (Fridovich, 1995). Three SODs are expressed by cells, each encoded by separate genes (reviewed in [Zelko et al., 2002]). Copper- and zinc-containing SOD (CuZnSOD, SOD1) is a homodimer found primarily in the cytoplasm (Keele Jr. et al., 1971; McCord and Fridovich, 1969), while small amounts have been discovered in the intermembrane space of mitochondria (Okado-Matsumoto and

Fridovich, 2001; Weisiger and Fridovich, 1973). Extracellular SOD (ECSOD, SOD3) has 40–60% amino acid homology with CuZnSOD, contains both copper and zinc in its active site, but is a membrane-bound enzyme in the extracellular region of the cell (Folz and Crapo, 1994; Hjalmarsson et al., 1987). MnSOD (SOD2) is a manganese-containing homotetramer (Borgstahl et al., 1992; Ravindranath and Fridovich, 1975; Wispe et al., 1989) found solely in the mitochondrial matrix (Okado-Matsumoto and Fridovich, 2001; Weisiger and Fridovich, 1973).

Hydrogen peroxide is a non-radical ROS, and a cell contains many enzyme systems that decompose hydrogen peroxide to water and molecular oxygen (Andreyev et al., 2005). Mitochondria contain two forms of peroxiredoxin (PRX) (Oberley et al., 2001): PRX III (Chang et al., 2004; Shibata et al., 2003) and PRX V (Seo et al., 2000). Peroxiredoxins use thioredoxin to decompose hydrogen peroxide, producing water and oxidized thioredoxin. Thioredoxin reductase is used to regenerate reduced thioredoxin (Choi et al., 2002; Lee et al., 1999).

Glutathione peroxidase (GPX) is another important enzyme that breaks down hydrogen peroxide, with GPX1 (Esworthy et al., 1997) and phospholipid-hydroperoxide GPX (PHGPX) (Maiorino et al., 2003) localized to the mitochondrial matrix and inner membrane, respectively (Panfili et al., 1991). GPX uses glutathione (GSH) to reduce hydrogen peroxide to water, generating oxidized glutathione (GSSG), and glutathione reductase is used to regenerate GSH (Kelner and Montoya, 2000).

Another vital hydrogen peroxide scavenger is catalase (Chelikani et al., 2004; Zamocky et al., 2008). Controversy exists concerning the subcellular localization of catalase. Some investigators report localization of catalase to the nucleus, peroxisomes, and the sarcoplasm, but not mitochondria, in mice overexpressing catalase (Zhou and Kang, 2000). Other investigators, in contrast, have identified catalase in mitochondria (Salvi et al., 2007) and in the mitochondrial matrix (Radi et al., 1991b).

2.3. MnSOD is Essential for Aerobic Life

Abundant evidence from various model systems demonstrates the critical role of MnSOD in protecting aerobic life from the lethal effects of atmospheric oxygen. Gregory and Fridovich (Gregory and Fridovich, 1973) found that *E. coli* B cells grown under 100% oxygen were resistant to the toxic effects of hyperbaric oxygen (20 atm) compared to *Bacillus subtilis* or *E. coli* B cells grown under normal atmosphere because of oxygen-stimulated MnSOD expression. These *E. coli* B cells were also more resistant to the superoxide-generating antibiotic streptonigrin. Experiments conducted with the yeast strain *Saccharomyces cerevisiae* var. *ellipsoideus* showed similar results (Gregory et al., 1974). Changes in MnSOD expression and/or activity can have dramatic effects on higher organisms, as well, and these changes are detailed below.

2.3.1. Effects of MnSOD on Development—Loss of MnSOD enzymatic activity through expression of inactive mutants or complete knockout of the *SOD2* gene resulted in early death in *Drosophila* (Duttaroy et al., 2003) and mouse (Li et al., 1995) models due to a decrease in the activity of a multitude of mitochondrial proteins (Li et al., 1995; Paul et al.,

2007) (see MnSOD Affects Mitochondrial Function, below). MnSOD is vital for viability in adult *Drosophila*, but knockout of MnSOD had no effect on embryogenesis, later development, or ifferentiation (Mukherjee et al., 2011), consistent with mouse models demonstrating that homozygous MnSOD knockout mice are normal size at birth and have no gross deformities (Li et al., 1995). Increased death rate seen in MnSOD homozygous knockout mouse neonates compared to wild-type and heterozygous MnSOD knockout littermates may be due to the inability of these mice to compensate for the high atmospheric levels of molecular oxygen compared to uterine O_2 levels. Interestingly, overexpression of CuZnSOD did not compensate for neonatal lethality resulting from diminished MnSOD expression, indicating that intracellular localization of antioxidant enzymes is important for modulation of ROS-mediated cellular damage (Copin et al., 2000).

Complete knockout of MnSOD in different genetic backgrounds can have strain-specific consequences. Huang *et al*. developed homozygous MnSOD knockout mice ($MnSOD^{-/-}$) in a C57BL/6J background (B6<*Sod2*−/−>), a DBA/2J background (D2<*Sod2*−/−>), and a cross of the two backgrounds (B6D2F1<*Sod2*−/−>). The researchers found a significant difference in the lifespan of $MnSOD^{-/-}$ depending on the background. Most of the B6<*Sod2*−/−> mice died *in utero* at embryonic day 15, and the mice that survived to birth were 21% smaller than $MnSOD^{+/+}$ littermates and died within 24 h. The B6<*Sod2*−/−> mice developed dilated cardiomyopathy prenatally. On the other hand, D2<*Sod2*−/−> and B6D2F1<*Sod2*−/−> mice survived to birth, had a mean life span of 8.7 d and 15.7 d, respectively, and had only mild hypertrophy of the heart compared to the B6<*Sod2*−/−> mice. The D2<*Sod2*−/−> developed severe metabolic acidosis and had an accumulation of fat in the liver compared to the B6D2F1<*Sod2*−/−> mice. B6D2F1<*Sod2*−/−> mice, however, became increasingly ataxic and suffered from frequent seizures. The phenotypic differences among the strains studied may be due to their having different compensatory mechanisms (Huang et al., 2001). One genetic modifier that may explain the strain-specific differences with MnSOD knockdown is mitochondrial NADPH transhydrogenase (NNT). $MnSOD^{-/-}$ mice in the C57BL/6J background are homozygous for a truncated *Nnt* allele (Nnt^{T}). When a normal copy of the *Nnt* allele from wildtype mice (Nnt^{W}) was introduced into the $MnSOD^{-/-}$ mice, cardiac function was preserved; heart failure was delayed; and the mice survived to the end of gestation. However, there was no increase in postnatal survival (Kim et al., 2010a).

ROS and RNS are vital for normal embryonic development (recently reviewed in [Ufer et al., 2010])]), and oxidative stress experienced by the embryo can lead congenital defects (reviewed in [Ornoy, 2007]). Therefore, it is not surprising that MnSOD is important for proper embryogenesis. Yon *et al*. (Yon et al., 2011) discovered an interesting dynamic in the expression of MnSOD in both embryonic and extra-embryonic tissues during the mouse gestational period studied (embryonic days (ED) 7.5–18.5). In extra-embryonic tissues, MnSOD expression at the mRNA and protein levels was elevated compared to embryonic tissues. MnSOD mRNA in extra-embryonic tissues was elevated from ED7.5–12.5, with a steady decline after ED13.5, while there was a gradual increase in MnSOD mRNA in embryonic tissues throughout the entire gestational period studied, with a similar pattern observed for MnSOD protein levels. The authors suggest that the high expression of

MnSOD in the extra-embryonic tissue may provide the embryo protection against oxidative stress during embryogenesis.

2.3.2. MnSOD and Aging—A life-long reduction in MnSOD expression can have important consequences on aging. In a study by van Remmen *et al*. (van Remmen et al., 2003), heterozygous knockout of MnSOD in mice led to a greater age-dependent increase in both nuclear and mitochondrial oxidative DNA damage (as measured by 8-oxodeoxyguanidine formation) in various tissues tested compared to wild-type mice. Surprisingly, there was no difference in life-span between $MnSOD^{+/-}$ and wildtype mice, and there were no statistically significant differences in various markers of aging tested between genotypes (cataract formation, carboxymethyl lysine and pentosidine levels in skin, and splenocyte proliferation). However, there was a 100% increase in cancer incidence in $MnSOD^{+/-}$ mice compared to wildtype, with a greater incidence of potentially fatal tumors (lymphoma, hemangioma, and adenocarcinoma).

Jang *et al*. studied the effects of MnSOD overexpression on age-related biomarkers. Elevated MnSOD led to an increase in aconitase activity, a decrease in lipid peroxidation, diminished age-related decline in mitochondrial ATP production, and it protected the mice from paraquat-induced oxidative stress. Interestingly, MnSOD overexpression did not have an effect on lifespan or age-related pathology (Jang et al., 2009). Overexpression of CuZnSOD, catalase, or the combination of CuZnSOD with either catalase or MnSOD had no effect on lifespan in mice (Perez et al., 2009). Conversely, MnSOD overexpression increased the mean life span in a *Drosophila* model without affecting overall oxygen consumption (Sun et al., 2002).

2.3.3. Tissue-Specific Effects of Altered MnSOD Expression

2.3.3.1. Cardiovascular System: Several studies have demonstrated the importance of MnSOD in proper cardiovascular system function. Early death of $MnSOD^{-/-}$ mice resulted from myriad anatomical abnormalities, including myocardial hypertrophy, reduced left ventricular wall thickness, and dilated left ventricular cavity, resulting in dilated cardiomyopathy (Li et al., 1995). Using a conditional knockout mouse model in which a lack of tetracycline results in reduced or complete loss of MnSOD expression (depending on genotype), Loch *et al*. discovered that life-long reduction in MnSOD led to serious cardiac defects, including heart hypertrophy, increased left ventricular internal diameter, and diminished fraction shortening and ejection fraction (Loch et al., 2009). Lack of MnSOD activity also results in diminished complex I and II activities, as well as significantly reduced aconitase activity (Li et al., 1995; Melov et al., 1999) and an increase in oxidative DNA damage in both cardiac and brain tissues (Melov et al., 1999). Loss of MnSOD can also affect the development of various cells comprising the blood, as demonstrated in a study by Lebovitz *et al*., where the researchers developed a MnSOD knockout mouse ($SOD2^{m1BCM}/SOD2^{m1BCM}$) by deleting both exons 1 and 2 from the *Sod2* gene. These mice were able to live up to three weeks after birth. However, the MnSOD knockout mice had hypocellular bone marrow due to diminished levels of all hematopoietic cells, resulting in severe anemia. These mice also developed cardiac injury, with roughly 10% of the mice

displaying cardiac injury because of ventricular wall thinning and balloon-like cardiac dilation (Lebovitz et al., 1996).

Work by this laboratory has focused on the cardiac and neurological toxicities of cancer chemotherapeutic drugs. For example, a major side effect of anthracycline chemotherapy drugs, such as adriamycin, is a dose-dependent cardiotoxicity (Simbre II et al., 2005) resulting in dilated cardiomyopathy and congestive heart failure (Minotti et al., 2004). Because mitochondria are targeted by adriamycin (Sarvazyan, 1996), and ROS production is an important mechanism of adriamycin-induced cardiotoxicity, modifications in the ROS-scavenging ability of cells can affect adriamycin-induced cardiac injury (Kang et al., 2002; Shioji et al., 2002; Sun et al., 2001). Using transgenic mice overexpressing MnSOD, this laboratory was the first to demonstrate the vital role of mitochondrial ROS in adriamycin-induced cardiac injury (Yen et al., 1996). Adriamycin treatment induced a significant reduction in the respiratory control ratio and state III respiration at complexes I and II in non-transgenic animals in contrast to mice overexpressing MnSOD, where only complex II was affected, suggesting that MnSOD protects complex I from deactivation resulting from adriamycin-induced superoxide production (Yen et al., 1999).

2.3.3.2. Nervous System: Alterations in MnSOD expression and/or activity can also affect the central nervous system. Loss of MnSOD activity resulted in a diminution of complex I, complex II, and aconitase activities in brain tissue (Li et al., 1995; Melov et al., 1999), as well as increased oxidative DNA damage (Melov et al., 1999). Flynn et al. discovered that synaptic termini isolated from the frontal cortex of $MnSOD^{-/-}$ mice had reduced mitochondrial spare respiratory capacity compared to $MnSOD^{+/+}$ mice. The $MnSOD^{-/-}$ mice compensated for the diminished oxidative phosphorylation by increasing the rate of glycolysis (Flynn et al., 2011). In the mouse model used by Lebovitz *et al.*, $MnSOD^{-/-}$ animals lived up to three weeks after birth and displayed multiple motor and behavioral abnormalities, including early onset of fatigue, circling behavior, and limb weakness that correlated with oxidative damage to nerves of the basal ganglia and brain stem (Lebovitz et al., 1996). Keller *et al*. found that overexpression of MnSOD protected PC6 pheochromocytoma cells from apoptosis induced by amyloid β-peptide, nitric oxide-generating compounds, and Fe^{2+} by inhibiting accumulation of peroxynitrite, nitration of proteins, and 4-hydroxynonenal. MnSOD overexpression prevented reduction in mitochondrial membrane potential caused by these apoptosis-inducing agents. Similar results were observed *in vivo* in MnSOD-overexpressing mice, where neuronal cell death, lipid peroxidation, and protein nitration after focal cerebral ischemia were significantly reduced compared to wildtype mice (Keller et al., 1998). MnSOD overexpression also protected PC12 pheochromocytoma cells and primary rat and mouse cortical cells from *N*-methyl-D-aspartate (NMDA) and nitric oxide-induced toxicity compared to controls. Cortical cells derived from MnSOD homozygous knockout were much more susceptible to NMDA and nitric oxide-induced cell death compared to MnSOD heterozygous knockout and wildtype cells, and overexpression of MnSOD in the homozygous cells conferred protection from NMDA and nitric oxide (Gonzalez-Zulueta et al., 1998). MnSOD overexpression in mice also decreased lesion volume compared to wildtype littermates after traumatic brain injury (Sullivan et al., 1999). Conditional knockout of MnSOD in postnatal

neurons did not increase oxidative damage but did result in increased disorganization of distal nerve axons after injury (Misawa et al., 2006). In an interesting study by Melov *et al*., treatment of MnSOD$^{-/-}$ mice with MnTBAP (manganese 5,10,15,20-tetrakis [4-benzoic acid] porphyrin) prevented cardiac dysfunction and early death associated with loss of MnSOD enzymatic activity. However, the MnTBAP-treated MnSOD$^{-/-}$ mice developed a movement disorder, eventually leading to complete debilitation. These mice demonstrated spongiform degeneration of the cortex and intramyelinic vacuolization characteristic of cytotoxic edema or neurological disorders associated with mitochondrial dysfunction, suggesting the MnTBAP was unable to cross the blood-brain barrier and revealing a vital role for MnSOD in normal brain function (Melov et al., 1998).

MnSOD also plays an important role in the development of seizures. A small subset of MnSOD heterozygous knockout (MnSOD$^{+/-}$) mice developed an age-dependent increased incidence of both spontaneous and handling-induced seizures correlating with decreased aconitase activity, oxidative mitochondrial DNA damage, and reduced mitochondrial utilization. These mice also demonstrated increased seizure susceptibility before any age-related effects occurred when given kainate. The MnSOD$^{+/-}$ mice also had an age-dependent decrease in glial glutamate transporter (GLT-1 and GLAST) expression, suggesting that the reduced ability to transport glutamate may be a mechanism of increased seizure incidence in this subset of mice (Liang and Patel, 2004). Similar effects on seizure incidence caused by MnSOD loss has been observed in MnSOD$^{-/-}$ mice, and treatment with the lipophilic metalloporphyrin AEOL 11207 significantly decreased the duration and frequency of the seizures but had no effect on severity. A potential side effect of cancer chemotherapeutic drugs is cognitive decline typified by decreased concentration, altered reaction time, and memory loss (Ahles and Saykin, 2007; Tannock et al., 2004). Referred to by patients as chemobrain (Nelson et al., 2007; Wefel et al., 2004), it is known in the literature as chemotherapy-induced cognitive impairment (CICI, recently reviewed in [Seigers and Fardell, 2011]). Adriamycin treatment can cause alterations in the structure (Brown et al., 1998; Inagaki et al., 2007) and activity (Silverman et al., 2007) of myriad regions of the brain. Oxidative stress is thought to be an important component of CICI (Joshi et al., 2005; Tangpong et al., 2007), and this laboratory was the first to show a unique mechanism of adriamycin-induced neurotoxicity involving TNF-α (Tangpong et al., 2006). Adriamycin is unable to cross the blood brain barrier (Ohnishi et al., 1995), but it has stimulated an increase in TNF-α in serum, whole brain homogenate, and elevated TNF-α staining in the hippocampus and cortex of mouse brain. Similar to the effects observed in cardiac tissue, adriamycin treatment resulted in a reduction in complex I activity leading to diminished state III respiration (Tangpong et al., 2006). Adriamycin also stimulated nitration, and deactivation, of MnSOD. The effects of adriamycin on MnSOD were not seen in iNOS knockout mice, suggesting a role for iNOS in adriamycin-induced neurotoxicity and also a vital part for MnSOD in prevention of CICI (Tangpong et al., 2007).

2.3.3.3. Liver: Kokoszka *et al*. used liver mitochondria isolated from wildtype and MnSOD$^{+/-}$ CD1 mice to study the effects of MnSOD loss on age-related changes in liver function. The researchers found an initial decrease in state IV respiration in young (5 months) MnSOD$^{+/-}$ mice compared to wildtype mice, with an increase in middle-aged (10–

14 months) and old (20–25 months) MnSOD$^{+/-}$ mice relative to wildtype controls. Surprisingly, there was a decrease in lipid peroxidation in old MnSOD$^{+/-}$ mice compared to controls. There was also a greater sensitivity to Ca^{2+}-dependent mtPTP at all ages in MnSOD$^{+/-}$ mice. An increase in apoptosis (as determined by TUNEL staining) and activities of all oxidative phosphorylation proteins (complex I, II, II+III, and IV) and citrate synthase were observed in the livers of MnSOD$^{+/-}$ mice compared to wildtype controls, suggesting a potential compensatory mechanism for diminished liver function (Kokoszka et al., 2001).

Interestingly, liver-specific knockout of MnSOD by crossing MnSOD-flox mice with albumin-Cre mice did not result in either changes in liver histology or function or oxidative damage (as determined by lipid peroxidation). These results are in contrast to other studies in mice with whole body knockout of MnSOD, which showed alterations in liver function (Kokoszka et al., 2001; Li et al., 1995) and morphology (Lebovitz et al., 1996), implying that the liver may contain compensatory mechanisms against oxidative stress or the liver is only susceptible to systemic oxidative stress (Ikegami et al., 2002).

2.3.3.4. Skeletal Muscle: Heterozygous knockout of MnSOD resulted in significant reduction in mitochondrial function in skeletal muscle in young mice (6–8 months) that mimics age-dependent decline in mitochondrial function, including ATP production, as well as complex I and IV activities (Mansouri et al., 2006). Specific knockdown of MnSOD in type IIB skeletal muscle using Cre/Lox technology resulted in a significant increase in mitochondrial superoxide production and oxidative damage, leading to decreased aerobic exercise capacity and a diminution of the gastrocnemius and extensor digitorum longus muscles to produce force over time (Lustgarten et al., 2009). Surprisingly, knockdown of MnSOD did not alter age-dependent muscle atrophy in type IIB skeletal muscle (Lustgarten et al., 2011).

2.3.3.5. The Immune System: Using a thymus-specific knockout of MnSOD, Case *et al*. found that a loss of MnSOD negatively affected T-cell development and modified adaptive immune system function due to an increase in apoptosis and T-cell developmental defects, resulting in increased susceptibility to influenza A H1N1 infection compared to control mice due to greater immunodeficiency. Treatment with Tempol or CTPO, both superoxide scavengers, rescued the MnSOD knockout mice from immunodeficiency, implying a role for MnSOD in sustaining adaptive immune response (Case et al., 2011).

2.3.3.6. Kidney: Interestingly, kidney-specific knockout of MnSOD did not affect the lifespan of the mice compared to Cre controls but did result in a significant reduction in body weight without changes in the weight of the vital organs tested (kidney, liver, heart, and lungs), various physiological parameters (blood glucose level and systolic blood pressure) or kidney function (as measured by serum creatinine). However, there were significant changes in kidney morphology, such as an increase in dilated distal tubules within the cortex, as well as swelling of the distal tubules and an increase in proteinacious casts within the lumen, compared to Cre controls. There was also a gene dose-dependent increase in tyrosine nitration of the cortical distal tubules and medullary regions of the kidney, suggesting an increase in oxidative stress in the kidney (Parajuli et al., 2011).

2.4. MnSOD Affects Mitochondrial Function

Changes in MnSOD expression/activity can have dramatic effects on mitochondrial function, including iron metabolism, apoptosis, and control of innate and adaptive immunity, among other activities (recently reviewed in [Holley et al., 2011]). Oxidative phosphorylation and the tricarboxylic acid cycle are two important functions of mitochondria affected by altered expression of MnSOD and may have an impact on the Warburg effect. Therefore, we will focus our attention on these two mitochondrial functions.

2.4.1. Electron Transport Chain—Complexes I, II, and III of the electron transport chain, while producers of superoxide, are potential victims of superoxide due to FeS centers in important subunits of these complexes (Albracht, 1980; Albracht and Subramanian, 1977; Ohnishi, 1975, 1998; Roessler et al., 2010; Teintze et al., 1982). Complexes I and III are also vulnerable to peroxynitrite-induced inactivation (Pearce et al., 2001). MnSOD knockdown in different model systems results in altered activities of complexes I, II, and III. Li *et al*. found that complete knockout of MnSOD led to a significant reduction in complex II activity in cardiac tissue (Li et al., 1995). Mitochondria from heterozygous MnSOD knockout mice had a diminished respiratory control ratio (RCR) compared to wildtype controls due to oxidation of the FeS center of complex I (Williams et al., 1998). In $MnSOD^{-/-}$ erythroblasts, expression of many nuclear gene-encoded subunits of all five oxidative phosphorylation complexes was downregulated compared to wildtype cells (Martin et al., 2011). Larosche *et al*. found an increase in inducible nitric oxide synthase (iNOS) expression in wildtype, $MnSOD^{+/-}$, and MnSOD overexpressing mice but an increase in nitration of complexes I and V only in $MnSOD^{+/-}$ mice (Larosche et al., 2010). Work by this laboratory found that in wildtype mice, treatment with the chemotherapeutic drug doxorubicin (a ROS generator) resulted in a significant reduction in RCR and state III respiration at complexes I and II in cardiac tissue, while overexpression of MnSOD protected complex I from doxorubicin-generated superoxide-induced deactivation (Yen et al., 1999).

2.4.2. Tricarboxylic Acid (TCA) Cycle—The TCA cycle is an important metabolic pathway in mitochondria, producing reducing equivalents that feed into the electron transport chain and generating various substrates used in a multitude of cellular functions. Changes in the activity of the enzymes of the TCA cycle are tied to different pathological conditions, including cancer (Briere et al., 2006; Rotig et al., 1997). Aconitase, which converts citrate to isocitrate (Briere et al., 2006), is vulnerable to ROS/RNS attack due to the presence of FeS centers in the enzyme (Cantu et al., 2009; Castro et al., 1994; Gardner et al., 1995; Han et al., 2005; Hausladen and Fridovich, 1994; Tortora et al., 2007). MnSOD is vital for preserving aconitase activity in many model systems, including *Drosophila* (Duttaroy et al., 2003), *Arabidopsis thaliana* (Morgan et al., 2008), yeast (Longo et al., 1999), and mouse (Melov et al., 1999; Williams et al., 1998). Aconitase activity is significantly reduced in liver mitochondria isolated from MnSOD heterozygous knockout mice but was rescued by addition of iron and dithiothreitol, suggesting inactivation of aconitase occurred through superoxide-induced protein oxidation and loss of iron (Williams et al., 1998). Using microarray analysis, Martin *et al*. found that complete knockout of MnSOD resulted in a significant reduction in the expression of almost all enzymes and

regulatory proteins involved in the TCA cycle (Martin et al., 2011). Overexpression of MnSOD in A549 human lung adenocarcinoma cells protected aconitase from hypoxia-reoxygenation-induced inactivation (Powell and Jackson, 2003), as well as electron transport chain inhibitors and phenazine methosulfate, a redox-cycling agent (Gardner et al., 1995).

2.4.3. Consequences for Energy Metabolism—Diminution of aconitase and succinate dehydrogenase activities resulting from loss of MnSOD expression and/or inhibition of enzyme activity may have far-reaching consequences on energy metabolism, especially with respect to the Warburg effect during the early stage of cancer development. For example, Li *et al*. reported multiple changes in metabolic markers in MnSOD$^{-/-}$ mice that correlate with decreased succinate dehydrogenase and aconitase activities, including decreased lactic acid and increased ketones in plasma, steatosis of the liver, and lipid deposition in skeletal muscle (Li et al., 1995). Similar results have been observed in mice with type IIb skeletal muscle-specific knockout of MnSOD, in which loss of MnSOD activity resulted in a significant reduction in aconitase (Lustgarten et al., 2009; Lustgarten et al., 2011) and succinate dehydrogenase (Lustgarten et al., 2011) expression and activity, correlating with greater exercise-induced glucose utilization and lactate production compared to wildtype animals (Lustgarten et al., 2011).

3. The Role of MnSOD in Cancer Development

MnSOD plays an important role in cancer development due to its ROS scavenging ability. Interestingly, MnSOD seems to have a dual role in the fate of cancer (recently reviewed in [Hempel et al., 2011]). Some studies demonstrate an elevation of MnSOD expression in cancer cells compared to surrounding normal tissue (Ho et al., 2001; Hu et al., 2007; Izutani et al., 1998; Janssen et al., 2000; Malafa et al., 2000; Toh et al., 2000; Tsanou et al., 2004). On the other hand, other studies have found that MnSOD expression is reduced in different cancers (Chuang et al., 2007; Cullen et al., 2003; Hu et al., 2005; Oberley and Buettner, 1979; Soini et al., 2001).

3.1. MnSOD as a Tumor Suppressor

While some studies highlight the tumor-supporting function of MnSOD in cancer, other studies demonstrate inhibition of many of the hallmarks of cancer by overexpression of MnSOD, including anchorage-independent cell growth and invasiveness (Behrend et al., 2005; Chuang et al., 2007; Liu et al., 1997; Venkataraman et al., 2005; Weydert et al., 2003). Church *et al*. provided the first study that characterized the tumor-suppressing effects of MnSOD. The researchers developed several clones of UACC-903 human melanoma stably expressing MnSOD and found that +psv$_3$neo vector controls and cells expressing antisense MnSOD mRNA grew quickly and had a morphology similar to the parental UACC-903 cells, as well as comparable expression of PCNA (a cell proliferation marker) and HMB-45 (a marker for melanoma). These cells also formed colonies in soft agar and tumors in nude mice. Consistently, overexpression of MnSOD in UACC-903 cells resulted in a more differentiated morphology, decreased colony formation in soft agar, diminished expression of both PCNA and HMB-45, and a complete failure for these cells to form tumors in nude mice (Church et al., 1993). Studies by this laboratory using FSa-II mouse

fibrosarcoma cells, which have undetectable levels of MnSOD, also demonstrated the tumor suppressing effects of MnSOD. Cell lines expressing the empty vector (pSV2-NEO) and different levels of MnSOD (low, moderate, and high) were developed, and mice were transplanted with these cells. While no morphological differences between tumors and metastases derived from parental, NEO, and MnSOD overexpressing cells were observed, there was a significant difference in the number of metastases that formed. Overexpression of MnSOD decreased the number of metastases in a dose-dependent manner compared to controls, indicating that MnSOD expression suppresses tumor aggressiveness (Safford et al., 1994). In another study using MnSOD-expressing Fsa-II cells, MnSOD expression significantly reduced the radiation dose needed to control one-half of the irradiated tumors compared to controls in mice transplanted with the cells (Urano et al., 1995).

Mechanisms by which MnSOD suppresses cancer growth include modulation of carcinogen-induced ROS levels (Oberley, 2005; Ridnour et al., 2004; Zhang et al., 2006) and sensitization of cancer cells to cell death induced by different ROS-generating agents *in vitro* and *in vivo* (Li et al., 1998). Overexpression of an active site mutant of MnSOD lacking product inhibition caused ROS-mediated growth retardation in HEK293 cells, which was impeded by catalase coexpression (Davis et al., 2004). In XR23M transformed x-ray immortalized rat embryonic fibroblasts, MnSOD overexpression decreased colony formation, diminished metastatic potential, and reduced tumor growth *in vivo* (Ridnour et al., 2004). In PC-3 human prostate cancer cells, overexpression of MnSOD decreased cell growth, correlating with increased hydrogen peroxide levels and mitochondrial membrane potential (Venkataraman et al., 2005).

Another important mechanism of MnSOD-mediated cancer suppression is induction of differentiation. Using the UACC-903 human melanoma cell line, Church *et al*. discovered that overexpression of MnSOD by introduction of (+)-sense MnSOD-5 cDNA resulted in a more differentiated morphology compared to empty vector controls (Church et al., 1993). Several reports using hepatocellular carcinoma cells lines and patient samples have revealed a direct correlation between the relative level of MnSOD expression and the degree of differentiation, with the poorly differentiated cell lines and tumors exhibiting lower expression of MnSOD compared to more well-differentiated cell lines and tumors (Aida et al., 1994; Galeotti et al., 1989; Yang et al., 2005). Using FSa-II mouse fibrosarcoma cells, this laboratory demonstrated that overexpression of MnSOD not only decreased proliferation and colony formation but also induced differentiation through inhibition of AP-1 DNA binding activity and AP-1 target gene expression (Kiningham and St. Clair, 1997). MnSOD overexpression also inhibited 5-azacytidine-induced apoptosis and enhanced differentiation by activating NF-κB and ERK MAP kinase (Zhao et al., 2001a).

In this laboratory, a major focus has been to obtain a deeper understanding of the importance of MnSOD in oxidative stress-induced tumor initiation/promotion. This laboratory was the first to report that protecting mitochondria from oxidative stress by overexpression of MnSOD inhibits neoplastic transformation. Using C3H 10T1/2 mouse embryonic fibroblasts expressing an empty vector or MnSOD-encoding vector, St. Clair *et al*. found that MnSOD overexpression protected the cells from neoplastic transformation induced by ionizing radiation but not the DNA intercalating agent 3-methylcholanthrene, suggesting the

importance of ROS in ionizing radiation-induced tumorigenesis and the impact of MnSOD in cancer prevention (St. Clair et al., 1992). Overexpression of MnSOD in C57BL/6 mice reduced papilloma incidence and multiplicity caused by the DMBA (7,12-dimethylbenz(a)-anthracene)/TPA (12-O-tetradecanoylphorbol-13-acetate) treatment course for tumor initiation and promotion by inhibiting TPA-induced oxidative stress compared to wildtype mice (Zhao et al., 2001b).

Since overexpression of MnSOD inhibited DMBA/TPA-induced tumorigenesis, it was thought that knockdown would enhance tumor formation. A similar number of papillomas was observed in both wildtype and MnSOD heterozygous knockout C57BL/6 mice after DMBA/TPA treatment due to an increase in proliferation and apoptosis in the basal layer of the epidermis. Despite the similarity in papilloma formation between the two genotypes, there was an increase in oxidative stress in the MnSOD heterozygous knockout mice as measured by an increase in oxidized proteins (Zhao et al., 2002). Later, it was discovered that apoptosis preceded proliferation in the basal layer of the epidermis, with apoptosis peaking at 6 h post-TPA treatment and mitosis peaking at 24 h post-TPA. Less proliferation was observed in wildtype mice compared to MnSOD heterozygous knockout mice. Treatment with the MnSOD mimetic MnTE-2-PyP^{5+} 12 h after each TPA treatment led to a significant diminution in proliferation and protein oxidation without affecting apoptosis, leading to a 50% reduction in tumor incidence compared to DMBA/TPA alone. The results from this study suggest that oxidative stress is an important early event in cancer development and indicate a potential mechanism of MnSOD inhibition of cancer formation (Zhao et al., 2005a).

3.2. MnSOD Facilitates Tumor Progression

Several studies demonstrate that increased expression of MnSOD can lead to augmented aggressiveness, growth, and survival of cancer cells. Palazzotti *et al*. discovered that overexpression of MnSOD in HeLa cervical carcinoma cells protected the cells from growth inhibition and cell death caused by serum deprivation. The researchers also discovered that in wildtype HeLa cells, serum starvation did not affect MnSOD expression. However, in HT29 colon carcinoma cells, serum starvation stimulated MnSOD expression and was associated with resistance to serum deprivation (Palazzotti et al., 1999). MnSOD overexpression protected HCT116 colon cancer cells from tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by inhibiting cytochrome *c* and Smac/DIABLO release from mitochondria (Mohr et al., 2008).

MnSOD expression is also associated with the metastatic behavior of cancer cells. Salzman *et al.* found that a significant increase in MnSOD activity occurred in patients with higher stages of head and neck squamous cell carcinoma, and also in those with locoregional metastases (Salzman et al., 2007). In gastric cancer, MnSOD expression was significantly higher in cancers with lymph node metastases compared to non-metastatic cancer (Malafa et al., 2000), as well as colorectal cancer (Nozoe et al., 2003). MnSOD expression is important for the metastatic behavior of MDA-MB-231 human breast cancer cells, an estrogen-independent cell line (Kattan et al., 2008). Expression of MnSOD increased after progestin treatment in the T47D human breast cancer cell line and was linked to progestin stimulation

of invasion in these cells, providing a potential mechanism by which progestins increase the aggressiveness of breast cancer cells (Holley et al., 2009). A potential mechanism of MnSOD-promoted metastasis is postulated to be an increase in hydrogen peroxide-dependent matrix metalloproteinase expression (Nelson et al., 2003; Ranganathan et al., 2001).

While many reports indicate that ectopic expression of MnSOD correlates with differentiation of cancer cells to less aggressive phenotypes (see section 3.2 above), others demonstrate that MnSOD expression leads to a less differentiated state. For example, Landriscina *et al.* studied MnSOD expression in 33 brain tumors of neuroepithelial origin with different degrees of differentiation and discovered an inverse correlation between MnSOD expression and the degree of differentiation, with the greatest level of MnSOD expression in the most undifferentiated tumors (Landriscina et al., 1996). Neuroendocrine differentiation is an important step in the progression of prostate cancer from an androgen-dependent to an androgen-independent state (Vashchenko and Abrahamsson, 2005), and MnSOD expression is associated with neuroendocrine differentiation in prostate tumor cells (Quiros et al., 2009). Overexpression of MnSOD stimulated androgen independence and neuroendocrine differentiation, and increased survival against cell death induced by TNF, docetaxel, and etoposide in LNCaP human prostate cancer cells (Quiros-Gonzalez et al., 2011).

3.3. MnSOD is a Double-Edged Sword in Cancer

Based on the seemingly inconsistent reports of MnSOD as a tumor suppressor and a tumor supporter, the question remains of what is the precise role of MnSOD in cancer? Does MnSOD suppress cancer or does it promote aggressiveness? To address this cancer dichotomy, we propose to consider how MnSOD affects hydrogen peroxide production. A report by Buettner *et al*. demonstrated that MnSOD affects the steady-state levels of hydrogen peroxide in systems where the equilibrium constant for superoxide production (K) is less than 1. In this case, the rate constant for the conversion of superoxide back to molecular oxygen is greater than the rate constant for the conversion of molecular oxygen to superoxide. This is a condition observed for the production of superoxide by the electron transport chain (for example, the coenzyme Q system). When MnSOD is present in the system, there is an increase in hydrogen peroxide production with increasing levels of MnSOD, with the greatest effects observed at low levels of MnSOD. However, when MnSOD levels are sufficiently high, there is only a moderate further change in hydrogen peroxide production. This increase in hydrogen peroxide occurs because MnSOD is drawing the equilibrium of the system to the right, driving increased superoxide production because a small amount of superoxide is being consumed to produce hydrogen peroxide and is not participating in the reverse reaction, in accordance with Le Chatelier’s principle. (Buettner et al., 2006).

This change in hydrogen peroxide flux with alterations in MnSOD levels can have far-reaching implications in cancer progression. In the early stages of cancer progression, when MnSOD levels are low (Oberley and Buettner, 1979), overexpression of MnSOD may inhibit cancer cell growth through myriad mechanisms due to increased hydrogen peroxide

flux (Li et al., 2000). Later in cancer progression, when the cells exhibit increased glucose and hydrogen peroxide metabolism and suffer from chronic oxidative stress (Biaglow and Miller, 2005; Pani et al., 2010; Powis and Kirkpatrick, 2007), increased expression of MnSOD may be beneficial to cancer cells by stimulating metastatic behavior (Connor et al., 2007; Hempel et al., 2011; Holley et al., 2009; Nelson et al., 2003).

Work by this laboratory is just now pulling back the veil that has obscured the nuanced role of MnSOD in cancer development. This laboratory recently reported the dual nature of MnSOD in the DMBA/TPA two-stage model of skin cancer development in a unique mouse model expressing a MnSOD promoter-linked luciferase reporter gene (Dhar et al., 2011). Treatment with DMBA, followed by repeated treatments with TPA over a 25 week period, resulted in a significant reduction in MnSOD luciferase reporter gene activity, as well as diminished MnSOD mRNA, protein, and enzyme activity in both DMBA/TPA-treated skin and in papillomas, compared to DMSO-treated controls. The observation period after DMBA/TPA treatment was extended to 48–60 weeks to allow for squamous cell carcinoma (SCC) formation, a more aggressive tumor type. Interestingly, MnSOD expression significantly increased at the luciferase reporter gene activity, mRNA, protein, and enzyme activity levels in the transition from papilloma to SCC.

The differences in MnSOD expression in the transition from papilloma to SCC are due to differential transcription factor binding activity on the *Sod2* promoter. The DNA binding activity of Sp1, which is vital for basal expression of MnSOD (Xu et al., 2002), is diminished in DMBA/TPA-treated normal appearing skin, with a further decrease in papilloma. In SCC, however, Sp1 DNA binding activity returned to nearly the same level as DMSO-treated skin control (Dhar et al., 2011). The tumor-suppressing transcription factor p53 also regulates MnSOD expression, in part, through its interaction with Sp1 (Dhar et al., 2006; Dhar et al., 2010). p53 DNA binding activity remained similar to DMSO-treated skin in both DMBA/TPA-treated skin and papilloma but was significantly reduced in SCC. To test the importance of Sp1 and p53 in MnSOD expression, p53 was knocked down by siRNA, and Sp1 overexpression was performed in the JB6 mouse epithelial cell line. Knockdown of either p53 or Sp1 overexpression alone increased MnSOD luciferase reporter gene activity and MnSOD protein expression, and simultaneous knockdown of p53 and overexpression of Sp1 had an additive effect (Dhar et al., 2011).

These results reveal the significance of MnSOD in both early- and late-stage cancer. MnSOD expression decreases as cells transition to early cancer, demonstrating the tumor-suppressing role for MnSOD, while MnSOD expression increases in the conversion from early, nonaggressive cancer to a more aggressive phenotype, showing the importance of MnSOD as a supporting protein in later stages of cancer. Because of the role of MnSOD as a ROS-scavenging enzyme, and the importance of ROS in the initiation of the Warburg effect (discussed below), it is attractive to suggest that changes in MnSOD expression may impact the metabolic switch from oxidative phosphorylation to the Warburg effect (Dhar and St. Clair, 2012).

4. The Warburg Effect in Cancer

Cells use multiple metabolic processes to generate energy for use in maintaining homeostasis. Oxidative phosphorylation is a mitochondrial energy-producing process by which electrons are transferred from NADH or $FADH_2$ to molecular oxygen to form water using four protein complexes (three of which are proton pumps [complexes I, III, and IV], and the other [complex II] ties oxidative phosphorylation to the TCA cycle), generating a proton gradient. The proton gradient is used to drive ATP synthesis at a fifth protein complex (ATP synthase). Another important energy-generating process is glycolysis, whereby one molecule of glucose is metabolized to two molecules of pyruvate, generating two net ATP molecules in the process. Pyruvate can then be further metabolized aerobically to CO_2 through oxidative phosphorylation or anaerobically to lactate (Berg et al., 2002). While they are separate processes, glycolysis and oxidative phosphorylation are also tied together at myriad points (summarized in Figure 1).

The metabolic switch from oxidative phosphorylation to glycolysis in the presence of oxygen, known as the Warburg effect, is an important hallmark in cancer (Warburg, 1956). The importance of glycolysis in cancer is evidenced by the overexpression of at least one enzyme in the transport or utilization of glucose in over 70% of cancers worldwide, representing 24 classes of neoplasia (Altenberg and Greulich, 2004). Warburg proposed that the increase in glycolysis in cancer cells was due to damage to respiration (Warburg, 1956). Interestingly, cancer cells continue to consume oxygen at rates similar to normal cells (Chance and Castor, 1952; Chance and Hess, 1959; Warburg et al., 1924). Koppenol *et al*. suggested that cancer cells do not necessarily undergo the Warburg effect because of damage to oxidative phosphorylation, although mutations in mitochondrial and nuclear genes encoding citric acid cycle and electron transport enzymes are reported in cancer. Rather, dysregulation of the expression of glycolytic enzymes due to activation of various tumor-promoting genes and inactivation of several tumor-suppressing genes occurs in cancer (Koppenol et al., 2011). The factors that contribute to activation of glycolysis in cancer cells include altered expression/activation of oncogenes and signal transduction pathways (Levine and Puzio-Kuter, 2010) like Myc (Dang et al., 2009), AKT (Pelicano et al., 2006), NF-κB (Levine and Puzio-Kuter, 2010), p53 (Madan et al., 2011), Src, and hypoxia-inducible factor HIF (Semenza, 2007)), as well as nuclear and mitochondrial DNA damage (Chen et al., 2007).

4.1. The Advantages of the Warburg Effect in Cancer

Glycolysis gives cancer cells many advantages over normal cells, even if energy production by glycolysis is not as efficient as oxidative phosphorylation. López-Lázaro suggests that cancer cells make the metabolic switch to glycolysis by diverting oxygen metabolism away from oxidative phosphorylation, decreasing ATP production and removing the allosteric inhibition of phosphofructokinase by ATP, thus increasing glucose metabolism by glycolysis (Lopez-Lazaro, 2008). Glucose and its metabolites provide the backbone for synthesis of nucleotides, lipids, and amino acids, all vital components for proliferation (Koppenol et al., 2011; Lopez-Lazaro, 2008; Moreno-Sanchez et al., 2007). Gatenby and Gillies posit that increased glycolysis in cancer cells may be an adaptive response to

intermittent hypoxia/anoxia that develops in the tumor during early stages of growth, which persists even in the presence of oxygen (Gatenby and Gillies, 2004), indicating the importance of the tumor microenvironment in metabolism. Glycolysis also increases lactate levels, creating an acidic environment that stimulates cancer growth and metastasis (Bonuccelli et al., 2010; Hirschhaeuser et al., 2011). Another advantage of aerobic glycolysis is increased production of reducing equivalents, such as NADPH, to regulate ROS levels. Aerobic glycolysis also increases resistance of cancer cells to apoptosis (reviewed in [Fogg et al., 2011]). Vaughn and Deshmukh found that glucose metabolism in cancer cells increased levels of GSH, which maintained cytochrome *c* in a reduced state and inhibited cytochrome *c*-induced apoptosis (Vaughn and Deshmukh, 2008). Ward and Thompson suggest that glycolysis may also provide various metabolites that drive oncogenic gene expression through both genetic and epigenetic mechanisms, such as activation of HIF-1 and Myc transcription factors and altered histone methylation, as well as stimulation of different signal transduction pathways, such as the PI3K/Akt/mTORC1 pathway, that all contribute to altered mitochondrial metabolism, leading to increased anabolic cell growth (Ward and Thompson, 2012).

A discussion of the Warburg effect in cancer cells is not complete without also considering the contribution of glutamine to the overall metabolic changes observed in cancer cells. Glutamine is the most abundant plasma amino acid and is a major nitrogen carrier. Glutamine, like glucose, is important for ATP production and provides intermediates for biosynthetic pathways. Glutamine is involved in nucleotide and hexosamine synthesis, the production of non-essential amino acids (DeBerardinis and Cheng, 2010), and is vital for GSH and NADPH production to protect against deleterious ROS levels (DeBerardinis and Cheng, 2010; Shanware et al., 2011). For example, Zhou *et al*. used mass spectrometry to identify increased expression of glycolytic enzymes in the PANC-1 pancreatic ductal adenocarcinoma cell line compared to normal pancreatic duct cells, concomitant with an increase in the expression of enzymes involved in glutamine metabolism (Zhou et al., 2012). In a paper by DeBerardinis *et al*., researchers reported their discovery that glutamine supplied the carbon needed for the utilization of glucose-derived carbon in biosynthesis reactions, especially fatty acid synthesis. Glutamine metabolism also provided the substrates needed to generate NADPH and to restore oxaloacetate levels for flux through the TCA cycle to provide the reactive intermediates needed for continued cell proliferation (DeBerardinis et al., 2007).

4.2. ROS and the Warburg Effect

ROS are important mediators in the development and progression of cancer by myriad mechanisms, including initiation of various signal transduction pathways, DNA damage, and activation of different transcription factors (Behrend et al., 2003; Gius and Spitz, 2006; Storz, 2005). Several studies suggest a critical role for ROS in stimulation of glycolysis in cancer cells. For example, Shi *et al*. discovered that human hepatoma cells were more resistant to hypoxia than normal hepatocytes, and this resistance correlated with an increase in glucose uptake and metabolism (as measured by lactate dehydrogenase [LDH] activity and lactate production), as well as an increase in ROS production and HIF-1α protein level. Overexpression of xanthine oxidase, which produces ROS, resulted in an elevation of

HIF-1α protein, hexokinase expression and LDH activity under normoxic conditions. Treatment with α-lipoic acid or overexpression of MnSOD attenuated glycolytic activity in the hepatoma cells, leading to decreased cancer cell growth *in vitro* and *in vivo*, confirming the importance of ROS in the regulation of the Warburg effect (Shi et al., 2009). The role of HIF-1 in the Warburg effect is discussed in more detail below.

While the Warburg effect is regulated by ROS, it can also affect ROS levels in cells. A recent study by Aykin-Burns *et al*. found that cancer cells generate constitutively higher levels of superoxide and hydrogen peroxide than nontumorigenic cells, which correlated with increased glycolysis in the cancer cells. The cancer cells were more susceptible to glucose deprivation-induced cell death and oxidative stress than nontumorigenic cells, and overexpression of either MnSOD or mitochondrially targeted catalase protected the cancer cells from glucose deprivation-induced cell death. Glucose deprivation also correlated with a significant reduction in NADPH, suggesting that increased glycolysis increases the generation of reducing equivalents to protect cancer cells from increased steady-state levels of ROS (Aykins-Burns et al., 2009).

5. A Role for MnSOD in Regulation of the Warburg Effect

5.1. Hypoxia-Inducible Factor (HIF)

HIF is a heterodimeric transcription factor composed of the constitutively expressed HIF-1β subunit and the O_2-regulated HIF-1α subunit (Wang and Semenza, 1995), and it is a member of the basic helix-loop-helix family of transcription factors (Jiang et al., 1996a). HIF-1 DNA binding activity increases with decreasing levels of O_2, with a maximal response in the 0.5% O_2 range (corresponding to ischemia/hypoxia *in vivo*) (Jiang et al., 1996b). HIF-1α stabilization is regulated by oxygen- and α-ketoglutarate-dependent hydroxylation catalyzed by prolyl-4-hydroxylase, recruiting pVHL to ubiquitinate HIF-1α and targeting it for degradation (reviewed in [Semenza, 2011]). HIF-1 regulates transcription of genes involved in a wide range of cellular functions, including iron metabolism, angiogenesis, cell proliferation, glucose metabolism, and apoptosis (reviewed in [Ke and Costa, 2006]).

HIF-1α is overexpressed in a wide range of cancers and in a substantial number of metastases, suggesting a vital role for HIF-1α in cancer development and progression (Zhong et al., 1999). For example, Robey *et al*. found that HIF-1α protein levels and glucose levels were lower in the nonmetastatic MCF-7 human breast cancer cell line compared to the more aggressive MDA-MB-435 and MDA-MB-231 cell lines (Robey et al., 2005). HIF-1 regulates expression in myriad genes involved in promoting the Warburg effect (Semenza, 2007, 2010; Stubbs and Griffiths, 2010), including increased expression of genes involved in glucose uptake (Ebert et al., 1995), glycolysis (Firth et al., 1995; Semenza et al., 1996), altering mitochondrial respiration (Fukuda et al., 2007; Kim et al., 2006; Papandreou et al., 2006) and stimulating mitochondrial autophagy (Bellot et al., 2009; Tracy et al., 2007; Zhang et al., 2008). HIF-1 can also cooperate with the oncogene c-Myc to regulate expression of glycolytic genes (reviewed in [Dang et al, 2009]).

Mitochondrial ROS are important for stabilization and activation of HIF-1α (reviewed in [Klimova and Chandel, 2008]). Hypoxia increased ROS generated from complex III of the electron transport chain, which correlated with an increase in HIF-1α protein levels and HIF-1 transcriptional activity. Loss of mtDNA or expression of catalase abrogated hypoxia-induced activation of HIF-1 (Chandel et al., 2000; Guzy et al., 2005; Patten et al., 2010). Bell *et al*. later identified the Q_o site of complex III as being important for HIF-1α stabilization (Bell et al., 2007). Alternatively, Chua *et al*. reported that HIF-1α stabilization is not dependent on complex III-generated ROS but does require an intact electron transport chain (Chua et al., 2010).

Numerous studies indicate an important role for MnSOD in regulation of HIF-1α protein stabilization and transcriptional activity. Using endothelial cells isolated from patients with idiopathic pulmonary arterial hypertension (IPAH-EC), Fijalkowska *et al*. found a higher level of HIF-1α protein and transcriptional activity under both normoxia and hypoxia than in control cells, which paralleled a decrease in MnSOD protein. Knockdown of MnSOD, but not CuZnSOD, in human umbilical vein endothelial cells resulted in increased HIF-1α protein under normoxia (Fijalkowska et al., 2010). Sasabe *et al*. found that knockdown of MnSOD slightly increased HIF-1α protein levels under normoxia but substantially increased HIF-1α protein under hypoxia through ROS-mediated mechanisms in oral squamous cell carcinoma cells. This MnSOD-dependent regulation of HIF-1α occurred at the transcriptional, translational, and post-translational levels. Knockdown of MnSOD diminished the interaction of HIF-1α with pVHL, resulting in a decrease in pVHL-dependent ubiquitination of HIF-1α. Loss of MnSOD also increased mRNA expression of HIF-1α under normoxia and hypoxia. A decrease in MnSOD also increased phosphorylation of p70S6K, 4E-BP1, and eIF-4E, proteins important for translation of HIF-1α protein. Inhibition of ERK and AKT activity by PD98059 and LY294002, respectively, abrogated the effects of MnSOD knockdown on HIF-1α protein levels (Sasabe et al., 2010). Knockdown of MnSOD in MCF-7 human breast cancer cells using siRNA resulted in an accumulation of HIF-1α under hypoxic conditions in a superoxide-dependent manner. Administration of various spin traps, the ROS scavenger Tempol, or the SOD mimetic AEOL10113 attenuated hypoxic-stimulated HIF-1α accumulation in MnSOD knockdown cells (Kaewpila et al., 2008).

MnSOD overexpression can also affect HIF-1α protein stability. Using MCF-7 cells expressing different levels of MnSOD enzyme activity, Wang *et al*. found that at either low or high levels of MnSOD activity, HIF-1α protein levels were elevated. However, at moderate levels of MnSOD activity (3–6 fold above parental cells), HIF-1α protein levels were not elevated. Expression and secretion of the HIF-1α target gene vascular endothelial growth factor correlated with changes in MnSOD activity and HIF-1α protein levels. Expression of mitochondria-targeted catalase or glutathione peroxidase-1 suppressed HIF-1α accumulation under hypoxia in MCF-7 cells with high MnSOD activity, implying that MnSOD-generated ROS may be important for HIF-1α accumulation in cells with high MnSOD activity (Wang et al., 2005).

The studies described above suggest a potential role for MnSOD in regulation of HIF-1α protein levels both in early phases of cancer development (when MnSOD expression is low)

and in later stages of cancer progression (when MnSOD expression can be elevated). How this biphasic effect of MnSOD on HIF-1α stabilization alters HIF-1α-dependent regulation of glycolytic enzymes, and, ultimately, the Warburg effect, is not yet known, but it presents a tantalizing target for potential cancer therapeutic targets in the future.

5.2. Mitochondrial DNA (mtDNA)

Mitochondria contain multiple copies of a circular double-stranded DNA molecule (16,569 bp) that contains 37 genes that encode for 22 tRNAs and 2 rRNAs that are vital for translation of the remaining 13 genes that encode various components of the electron transport chain (Falkenberg et al., 2007). Seven subunits for complex I are encoded by mtDNA, while one, three, and two subunits are encoded by mtDNA for complexes III, IV, and V, respectively. The remaining mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytosol, and then transported to mitochondria (Wallace, 2010). mtDNA is assembled into nucleoids containing 6–10 individual mtDNA genomes (Iborra et al., 2004; Legros et al., 2004), as well as proteins essential for synthesis and transcription of mtDNA, including mitochondrial single-stranded DNA binding protein (mtSSB), DNA polymerase γ (Polγ), and mitochondrial transcription factor A (Chen and Butow, 2005; Garrido et al., 2003; Legros et al., 2004). Mitochondrial function declines with age (Boffoli et al., 1994) and is linked to a decrease in mtDNA content and an increase in oxidative mtDNA damage (Short et al., 2005), contributing to a number of age-related conditions (Tanaka et al., 1996; Wallace, 2010), such as diabetes, Parkinson's disease, heart failure (Kang and Hamasaki, 2005) and cancer (Brandon et al., 2006; Lu et al., 2009).

mtDNA damage is caused by myriad agents, including ionizing radiation (Richter et al., 1988), ultraviolet light (Berneburg et al., 1997; Takai et al., 2006), and ROS (Richter et al., 1988; Takai et al., 2006; Williams et al., 1998) (Yakes and van Houten, 1997), with the D-loop region highly susceptible to oxidative damage (Mambo et al., 2003). mtDNA sequences that encode different complex I subunits are also prone to damage, and damage to these regions may contribute to increased superoxide production (Cortopassi and Wang, 1995). In one study, cells lacking mtDNA (ρ^0) cells or cells that express mtDNA with a 4977-bp deletion have greater ROS production compared to parental cells or cells that have wild-type mtDNA reintroduced (Indo et al., 2007). A malicious situation can occur in which mtDNA damage causes altered mitochondrial function, resulting in greater ROS production, which leads to more mtDNA damage (Birch-Machin and Swalwell, 2010).

Numerous mutations in mtDNA have been identified in samples of myriad cancer types and contribute to cancer development, in part, by increased ROS production, as well as altered activation of signaling pathways and modified susceptibility to apoptosis (reviewed in [Lu et al., 2009; Shen et al., 2010]). A study by Petros *et al*. revealed that many mtDNA mutations exist in prostate cancer cells. They also discovered a significant increase in mutations for the mtDNA genes encoding subunits of complex I in prostate cancer compared to non-cancer controls and the general population. To determine the effects of a mtDNA mutation in the ATP6 subunit, the researchers generated PC3 cybrid (cytoplasmic hybrid) cell lines in which the cell's mtDNA was replaced with either ATP6 wildtype (T8993T) or mutant (T8993G) mtDNA. The cells expressing the T8993G mtDNA grew much faster *in vivo*, indicating that

mtDNA mutations can contribute to cancer phenotypes (Petros et al., 2005). An interesting study by Ishikawa *et al*. using P29 and A11 mouse lung carcinoma cell lines derived from Lewis lung carcinoma that have low (P29) and high (A11) metastatic potential revealed that cybrid cell lines with A11 mtDNA had diminished complex I activity compared to cells containing P29-derived mtDNA, regardless of whether the nuclear DNA was from the P29 or A11 cell line. Cybrid cells with the A11 mtDNA also had greater metastatic potential both *in vitro* and *in vivo*. The mtDNA that conferred the greatest increase in metastatic potential contained a mutation in the gene encoding the ND6 subunit of complex I, resulting in enhanced ROS production. Treatment with N-acetylcysteine (NAC) suppressed metastatic potential, demonstrating the importance of mitochondrial ROS resulting from mtDNA damage in cancer aggressiveness (Ishikawa et al., 2008). Cybrid mouse fibroblasts containing mtDNA from BALB/cJ mice (mtBALB), which contain a somatic mutation in the mitochondrial $tRNA^{Arg}$ gene, exhibited increased growth rate, ROS production and migration/invasion compared to cybrid cells containing mtDNA from C57BL/6J mice, which were suppressed by administration of antioxidants vitamin E or NAC, again suggesting a link between mtDNA mutation-linked ROS production and tumor-like phenotype (Jandova et al., 2012). mtDNA mutations can either be heteroplasmic (mutant mtDNA coexisting with wildtype mtDNA) or homoplasmic (containing only the mutant mtDNA), and Park *et al.* demonstrated that cybrid cells that are heteroplasmic for mtDNA containing a mutation in the NADH dehydrogenase subunit 5 (*ND5*) gene of complex I were much more aggressive than cells homoplastic for the *ND5* mutation, with an increase in colony formation *in vitro* and tumor growth *in vivo* (Park et al., 2009a).

Several reports indicate that altered mtDNA integrity, either through mutations or changes in mtDNA copy number, contribute to the Warburg effect. Cybrid 143B cell lines containing various mtDNA DNA point mutations and deletions exhibited increased glycolysis as measured by increased lactate and pyruvate production. Extracellular lactate levels were inversely correlated with ATP production and oxygen consumption (Pallotti et al., 2004). Pelicano *et al*. found that ρ^0 leukemia (HL-60) and lymphoma (Raji) cell lines had a greater dependency on glycolysis than parental cells, increased AKT activation, and increased levels of NADH, leading to resistance to both hypoxia and cancer chemotherapeutic drugs (Pelicano et al., 2006). Sun *et al.* discovered that transfection of OKF6 immortalized keratinocytes and O19 head and neck squamous cell carcinoma cells with mtDNA containing a mutation of the ND2 subunit of complex I resulted in HIF-1α stabilization due to a decrease in pyruvate dehydrogenase (PDH) protein levels, increased PDH kinase 2 (PDK2) expression and phosphorylation of PDH, and enhanced ROS generation (Sun et al., 2009). Transfection of HeLa cells with NADH dehydrogenase subunit 2 (*ND2*) mutants identified from patients with head and neck squamous cell carcinoma resulted in increased anchorage-dependent and – independent cell growth, as well as greater ROS generation, glycolysis, and HIF-1α protein levels compared to vector and wildtype controls (Zhou et al., 2007).

MnSOD is important for protection of mtDNA from ROS-mediated damage. MnSOD associates with *E. coli* K-12 DNA (Steinman et al., 1994). Oxidative mtDNA damage increases with age but is much greater in MnSOD heterozygous knockout mice compared to

wildtype mice (van Remmen et al., 2003). MnSOD is expressed as an adaptive response to the loss of, or damage to, mtDNA (Garcia-Ramirez et al., 2008; Park et al., 2004). MnSOD overexpression protects mtDNA against damage induced by UV (Takai et al., 2006) and acute ethanol exposure (Larosche et al., 2010; Mansouri et al., 2010). Overexpression of MnSOD or MnSOD mimetic treatment inhibits mtDNA oxidation resulting from high glucose in bovine retina endothelial cells compared to glucose alone, resulting in increased expression of different electron transport chain components (Madsen-Bouterse et al., 2010). MnSOD is part of the nucleoid complex, interacting with Polγ, mtDNA, and glutathione peroxidase, and MnSOD may protect mtDNA from ROS-mediated damage (Kienhofer et al., 2009). Polγ is susceptible to oxidative inactivation, potentially affecting mtDNA replication and repair (Graziewicz et al., 2002). This laboratory confirmed the interaction of MnSOD with Polγ and mtDNA and revealed that MnSOD may be important for the protection of mtDNA from UV-induced damage by inhibiting UV-mediated nitration and inactivation of Polγ (Bakthavatchalu et al., 2011). The protection of mtDNA from damage through MnSOD-dependent mechanisms may provide a method for MnSOD regulation of the Warburg effect.

5.3. p53

The tumor suppressor p53, also known as the “guardian of the genome” (Lane, 1992), plays an important role in regulation of the Warburg effect (recently reviewed in [Madan et al., 2011]). p53 directly suppresses the expression of the glucose transporters GLUT1 and GLUT4. The region of the GLUT4 promoter that spans -66/+163 confers p53 responsiveness, and p53 directly binds to the GLUT4 promoter *in vitro* (Schwartzenberg-Bar-Yoseph et al., 2004) and indirectly regulates GLUT3 expression through inhibition of the IKK-NF-κB pathway (Kawauchi et al., 2008). The increase in aerobic glycolysis due to loss of p53 results in an increase of O-linked β-N-acetylglucosamine modification of IKKβ, leading to enhanced IKKβ catalytic activity and greater NF-κB-linked glucose metabolism (Kawauchi et al., 2009). p53 also affects the expression of glycolytic enzymes. Phosphoglycerate mutase (PGM), which catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate, is transcriptionally repressed by p53 (Kondoh et al., 2005). p53 upregulates expression of the fructose bisphosphatase TP53-induced glycolysis and apoptosis regulator (TIGAR), which inhibits phosphofructokinase-1 (PFK-1) activity by decreasing the amount of fructose-2,6-bisphosphate available for PFK-1. Simultaneously, TIGAR increases fructose-1,6-bisphosphatase (FBPase-1) activity, diverting glucose away from the glycolysis pathway and into the pentose phosphate pathway, leading to an increase in NADPH production and resulting in an increase in GSH levels and a decrease in ROS levels (Bensaad et al., 2006). Recently, parkin, a protein associated with Parkinson’s disease, was identified as a p53 target gene. Parkin is important for mediating the effects of p53 on glycolysis. Knockdown of p53 leads to reduced levels of parkin and increased levels of glycolysis, which was reversed by overexpression of parkin (Zhang et al., 2011).

Another important mechanism of Warburg effect regulation by p53 is inhibition of HIF-1 transcription. p53 competes with HIF-1 for binding to p300 (Blagosklonny et al., 1998). p53 also affects HIF-1 activity by regulating the expression of proline oxidase. Proline oxidase stimulates production of P5C, leading to glutamate and α-ketoglutarate production. α-

ketoglutarate is a vital substrate for prolyl hydroxylase, which catalyzes the hydroxylation of HIF-1α, targeting it for degradation (Liu et al., 2009).

p53 can also alter the Warburg effect by affecting mitochondrial respiration. SCO2 (synthesis of cytochrome *c* oxidase 2), which is important in the assembly of cytochrome *c* oxidase (complex IV of the electron transport chain), is an important target for p53 that enhances mitochondrial respiration (Matoba et al., 2006). Subunit I of cytochrome *c* oxidase can also be upregulated by p53 (Okamura et al., 1999). p53 also affects mtDNA, which can impact oxidative phosphorylation. Loss of p53 results in diminished mtDNA copy number, decreased mitochondrial mass, and a diminution of mitochondrial membrane potential (Lebedeva et al., 2009). Mitochondrial transcription factor A (TFAM) (which is important for mtDNA transcription and maintenance) (Park et al., 2009b) and p53-inducible ribonucleotide reductase (RRM2B/p53R2) (which supplies dNTPs for mtDNA synthesis) (Bourdon et al., 2007; Lebedeva et al., 2009) are important p53 transcriptional targets.

p53 also plays a more direct role in mtDNA maintenance. Using HCT116 colorectal cancer cells, Achanta *et al*. found that knockout of p53 rendered the cells more susceptible to mtDNA depletion induced by ethidium bromide (EtBr). In HCT116 cells expressing wildtype levels of p53, p53 translocates to mitochondria after EtBr treatment or exposure to rotenone (a complex I inhibitor that stimulates mitochondrial ROS production) to protect mtDNA from damage induced by EtBr or ROS (Achanta et al., 2005). Mitochondrial translocation of p53 is associated with adriamycin-induced mtDNA damage in murine cardiomyocytes (Nithipongvanitch et al., 2007) and brain tissue (Tangpong et al., 2007), and this cardiac damage is considerably greater in p53 homozygous knockout mice, suggesting p53 protects cardiomyocytes from adriamycin-imposed mitochondrial injury (Nithipongvanitch et al., 2007). Mitochondrial p53 inhibits incorporation of nucleoside analogs into mtDNA because p53 possesses intrinsic 3′→5′ exonuclease activity (Bakhanashvili et al., 2009). p53 interacts directly with human mitochondrial single-stranded binding protein (HmtSSB), enhancing the exonuclease activity of p53 (Wong et al., 2009). p53 also interacts with Polγ (Achanta et al., 2005) and stimulates both the nucleotide incorporation (Chen et al., 2006; de Souza-Pinto et al., 2004) and glycosylase activities (Chen et al., 2006) of Polγ, as well as the polymerization and 3′→5′ exonuclease activities (Achanta et al., 2005).

p53 affects mitochondrial ROS homeostasis and can also be affected by mitochondrial ROS. Using mouse neonatal fibroblasts (MNFs) derived from p53 wildtype and p53 null C57/B6 mice and primary human fibroblasts treated with p53 shRNA to knockdown p53, Lebedeva *et al*. found that loss of p53 resulted in reduced cellular and mitochondrial superoxide levels and a simultaneous increase in cellular hydrogen peroxide levels, as determined by MitoSox Red, dihydrofluorescein diacetate, and dihydroethidium staining and fluorescent-assisted cell sorting. While the study did not investigate the mechanisms of the changes in ROS levels, the researchers speculate that the decrease in superoxide production and increase in hydrogen peroxide may be due to the loss of p53-mediated inhibition on MnSOD activity, either through altered MnSOD gene expression or physical interaction of p53 with MnSOD (Lebedeva et al., 2009). p53 regulates expression of several important antioxidant enzymes, such as sestrins, apoptosis-inducing factor (AIF), aldehyde dehydrogenase 4 (ALDH4),

glutathione peroxidase 1 (Gpx1), and MnSOD (Brookes et al., 2004; Budanov et al., 2004; Hussain et al., 2004; Liu et al., 2008a; Sablina et al., 2005; Stambolsky et al., 2006). This laboratory has demonstrated the importance of p53 in blocking both constitutive and TPA-induced MnSOD gene expression due to interactions of p53 with the transcription factor Sp1 in the MnSOD promoter. Blockade of p53-mediated repression of MnSOD expression is achieved by expression of Sp1 mutants or knockdown of Sp1 by siRNA (Dhar et al., 2006). In JB6 mouse epithelial cells, TPA treatment resulted in both mitochondrial and nuclear translocation of p53, resulting in stimulation of MnSOD expression but no corresponding increase in MnSOD enzyme activity. Treatment with the MnSOD mimetic MnTE-2-PyP^{5+} had little effect on p53 mitochondrial translocation but completely blocked p53 nuclear localization and expression of the p53 target gene Bax. p53 coimmunoprecipitated with MnSOD, implying that this interaction may interfere with MnSOD enzyme activity (Zhao et al., 2005b). Treatment with cyclosporin A (a mitochondrial permeability transition pore inhibitor) inhibited TPA-induced changes in both mitochondrial membrane permeabilization and complex I activity and decreased p53 mitochondrial translocation (Liu et al., 2008b). These results suggest that altered mitochondrial ROS production can affect p53 cellular localization activity, which can feed forward to induce greater mitochondrial ROS production and alter the expression of p53-responsive genes that impact various cellular functions that may contribute to selection of cancer phenotypes, such as the Warburg effect.

5.4. Sirtuin 3 (SIRT3)

SIRT3 is a mitochondria-localized, NAD$^+$-dependent deacetylase (Michishita et al., 2005). SIRT3 is an important tumor suppressor vital for preserving mitochondrial integrity and inhibiting mitochondrial ROS production, as well as hindering the Warburg effect (Bell et al., 2011; Finley et al., 2011; Kim et al., 2010b). Bell *et al*. found that loss of SIRT3 increased HIF-1α transcriptional activity in both mouse embryonic fibroblasts and various cancer cell linesunder both 21% O_2 and hypoxic (1% O_2) conditions and identified complex III as the site of ROS production responsible for increased HIF-1α activity with loss of SIRT3 (Bell et al., 2011). Using SIRT3-null mouse embryonic fibroblasts (MEFs), Finley *et al*. found that loss of SIRT3 resulted in a metabolic shift toward glycolysis, as measured by an increase in glycolytic intermediates and a decrease in TCA cycle intermediates, compared to wildtype MEFs. This increase in glycolysis was due to stabilization and nuclear localization of HIF-1α and expression of HIF-1α target genes involved in both glucose uptake (*Glut1* and *Hk2*) and glucose metabolism (*Pdk1*, *Ldha*, and *Pgk1*), resulting in increased growth and transformation potential of SIRT3-null MEFs compared to wildtype MEFs. Interestingly, SIRT3 does not interact directly with HIF-1α. Instead, SIRT3 regulates HIF-1α stabilization by ROS-mediated mechanisms (Finley et al., 2011).

MnSOD undergoes different post-translational modifications that affect MnSOD enzyme activity (reviewed in [Yamakura and Kawasaki, 2010]). Recently, MnSOD was identified as a target of SIRT3. SIRT3-null MEFs had much lower levels of MnSOD enzyme activity compared to wildtype MEFs without any change in MnSOD protein levels. However, SIRT3-null MEFS had higher levels of acetylated MnSOD compared to wild-type MEFs. Reexpression of SIRT3 led to a decrease in MnSOD acetylation and restored MnSOD enzyme activity. SIRT3-dependent deacetylation of MnSOD at Lys122 was essential for

maintaining MnSOD activity (Tao et al., 2010). SIRT3-null MEFs produced greater amounts of ROS after ionizing radiation compared to wildtype MEFs, and this increase in ROS was diminished when SIRT3-null MEFs expressed a Lys122Arg MnSOD mutant incapable of being acetylated (Tao et al., 2010). These results suggest that the regulation of MnSOD activity by SIRT3 may be important for the effects of SIRT3 in response to nutrient status and different stressors (reviewed in [Ozden et al., 2011]). Given the role of HIF-1α in the regulation of glycolysis and the importance of SIRT3 in regulating HIF-1α transcriptional activity, it is tempting to speculate that MnSOD may be the lynchpin that ties these two important proteins together in the regulation of the Warburg effect.

6. Concluding Remarks

The Warburg effect is a vital metabolic change that occurs during cancer development, providing rapidly dividing cancer cells with the building blocks needed for synthesis of proteins, nucleic acids, and membranes. ROS are important regulators of the Warburg effect by modulation of different signal transduction pathways and transcription factors that control the expression of genes involved in various aspects of glycolysis. Simultaneously, the Warburg effect also protects cancer cells from the harmful effects of ROS by shuttling metabolites away from oxidative phosphorylation and by synthesis of myriad reducing equivalents. The mitochondrial antioxidant enzyme MnSOD has a dual role in cancer: acting as a tumor suppressor in early stage cancer and supporting cancer growth in later stages of cancer progression. Recent *in vivo* studies from this laboratory suggest that reduction of MnSOD may be an early ROS signal for the metabolic shift observed in cancer cells. Because changes in MnSOD expression and/or activity affect so many avenues by which cells regulate the Warburg effect, a persuasive argument can be made suggesting a link between MnSOD and the regulation of the Warburg effect (summarized in Figure 2). Time and effort will tell whether this link exists and whether this link can be exploited for the creation of novel treatments for numerous cancers, providing hope for countless cancer patients worldwide.

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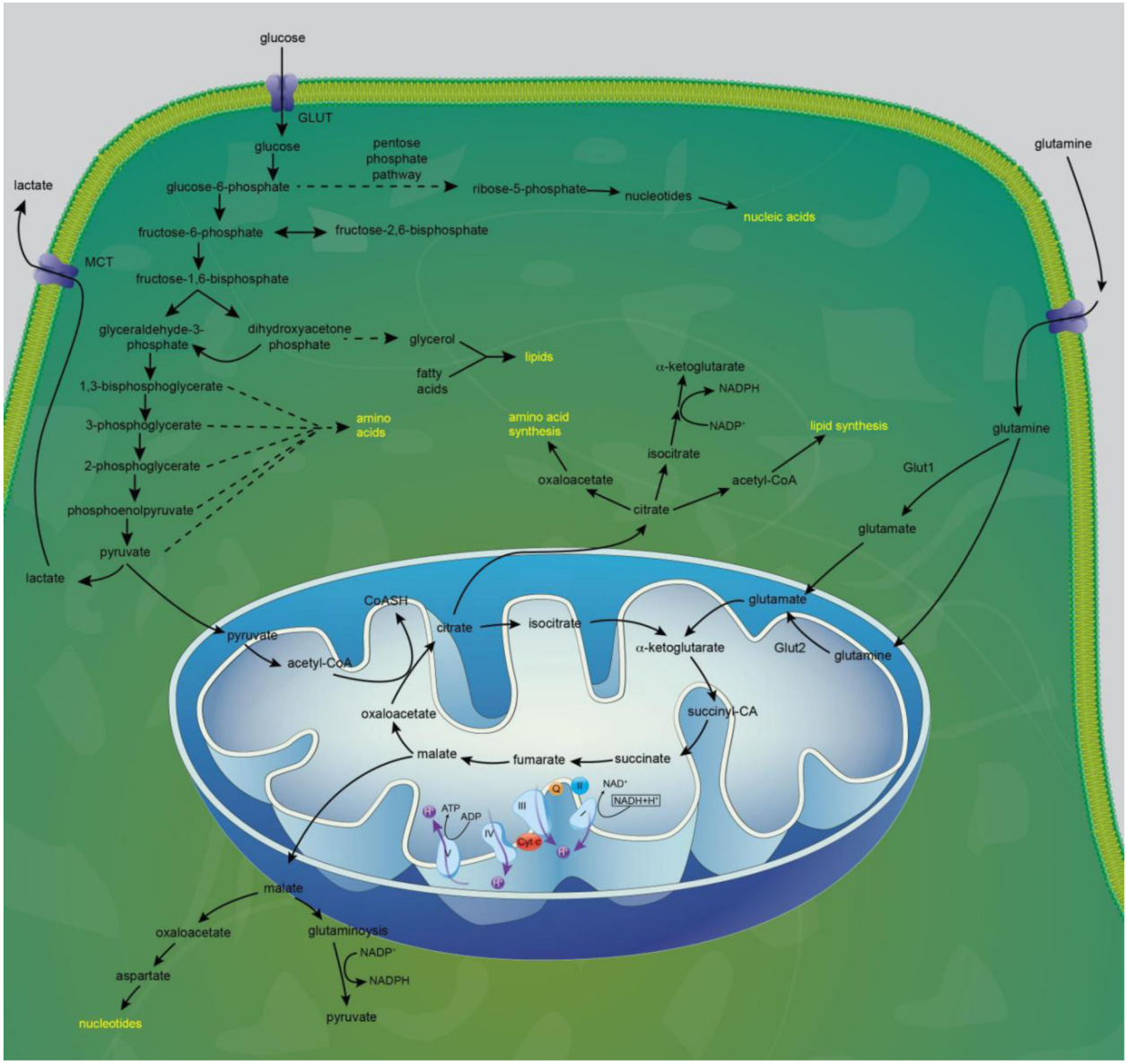

Figure 1.

Important metabolic pathways in normal and cancer cells. Glycolysis, the TCA cycle, and oxidative phosphorylation all contribute to both energy production in cells and generation of various components vital for synthesis of lipids, proteins, and nucleic acids necessary for cell division and maintenance.

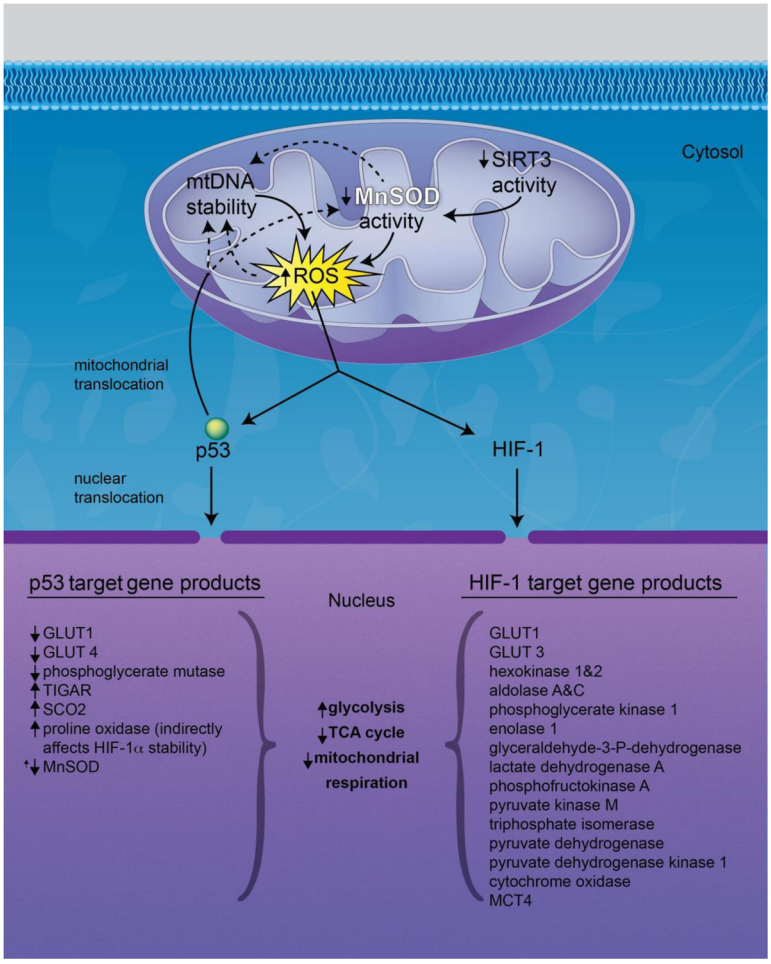

Figure 2.

Potential effects of altered MnSOD activity on the Warburg Effect in cancer cells. Changes that lead to a decrease in MnSOD activity result in elevated mitochondrial ROS, which can be detrimental to mtDNA stability, leading to a further increase in mitochondrial ROS levels and altered activity and localization of important transcription factors. Mitochondrial ROS can activate nuclear translocation of p53 and increase mitochondrial localization. Mitochondrial p53 can suppress MnSOD activity and contribute to increased mitochondrial ROS levels. Activation of p53 nuclear translocation may alter expression of several p53 target genes involved in metabolism. HIF-1 activity may also be affected by increased mitochondrial ROS, resulting in greater expression of HIF-1 target genes involved in various aspects of glycolysis. Changes in activity/localization of p53 and HIF-1 resulting from decreased MnSOD activity may ultimately contribute to the Warburg effect by increasing glycolysis and decreasing TCA cycle and mitochondrial respiration in cancer cells.