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Review Series

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Emerging evidence for targeting mitochondrial metabolic dysfunction in cancer therapy

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Mammalian cells use a complex network of redox-dependent processes necessary to maintain cellular integrity during oxidative metabolism, as well as to protect against and/or adapt to stress. The disruption of these redox-dependent processes, including those in the mitochondria, creates a cellular environment permissive for progression to a malignant phenotype and the development of resistance to commonly used anticancer agents. An extension of this paradigm is that when these mitochondrial functions are altered by the events leading to transformation and ensuing downstream metabolic processes, they can be used as molecular biomarkers or targets in the development of new therapeutic interventions to selectively kill and/or sensitize cancer versus normal cells. In this Review we propose that mitochondrial oxidative metabolism is altered in tumor cells, and the central theme of this dysregulation is electron transport chain activity, folate metabolism, NADH/NADPH metabolism, thiol-mediated detoxification pathways, and redox-active metal ion metabolism. It is proposed that specific subgroups of human malignancies display distinct mitochondrial transformative and/or tumor signatures that may benefit from agents that target these pathways.

Introduction

Mitochondria contain the oxidation/reduction (redox) O_2-consuming metabolic pathways necessary for cellular and organismal survival. The functions of these pathways include energy production and the removal and/or detoxification of damaging metabolites produced as by-products of oxidative phosphorylation (1–3). In this regard, mitochondria are constantly sensing energy availability, as well as the cell's current energy requirements, to ensure that the activities of the electron transport chain (ETC) and tricarboxylic acid (TCA) cycle match the cellular need for energy consumption (4–6). In addition, mitochondria also regulate the antioxidant enzymes and signal transduction pathways that lead to the detoxification of reactive by-products formed during energy production (7, 8). This regulatory balance is referred to as metabolic reprogramming, and it directs mitochondrial homeostasis.

The mitochondria in mammalian cells have evolved to monitor and maintain the integrity of mitochondrial function, as well as communicate and coordinate functions in other cellular organelles (9–11). In this regard, it is well established that disrupted normal mitochondrial function creates a cellular environment that is permissive for a host of human illnesses in both children and adults (12–14). In adults, a decline in mitochondrial quality and activity is more commonly observed with increasing age and appears to play a significant role in degenerative diseases associated with aging, including cardiovascular disease, neurodegenerative diseases, insulin resistance, carcinogenesis, and tumor cell resistance to therapy (15–19).

One of the most studied and debated mitochondrial metabolic abnormalities in human tumors is commonly referred to as the Warburg effect, but in the past few years this process has been shown to be more complex than simply an increase in glycolysis. In this regard, we now know that mitochondrial energy pathways entail changes in the regulation of both glycolysis and mitochondrial respiration, including oxidative phosphorylation, as compared with normal tissue, as well as higher flux through both of these pathways (20–22). However, attempts to target these processes in order to enhance anticancer therapy responses have not led to clinically applicable therapies (23, 24). In addition to changes in carbon flux, cancer cells tend to also exhibit significant increases in the production of superoxide ($O_2^{.-}$) and hydrogen peroxide (H_2O_2), both of which can induce prooncogenic signaling pathways as well as directly damage cellular micromolecules (22, 25).

In this regard, tumor cells often demonstrate altered activity of mitochondrial enzymes that could compensate for changes in tumor cell ROS levels and downstream damage products by upregulating the activity of antioxidant defenses (25, 26). These observations, coupled with advances in metabolomic analysis and carbon tracing, provide tools to elucidate the underlying basis for mitochondrial metabolic abnormalities in cancer. These technologies have also advanced our understanding of the role of redox biology in cancer initiation, progression, and metastasis, as well as strengthening the hypothesis that aberrant mitochondrial oxidative pathways represent potential molecular targets for the development of specific therapeutic interventions (7, 22, 25, 27–29).
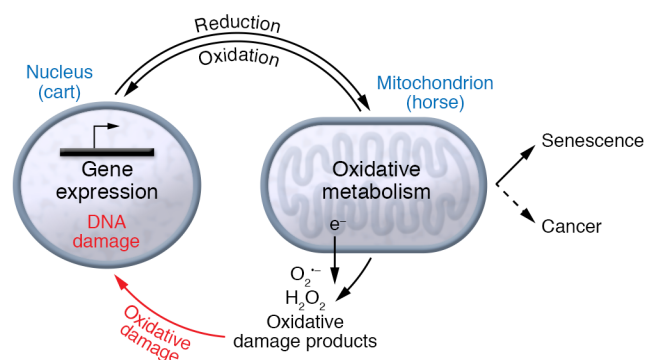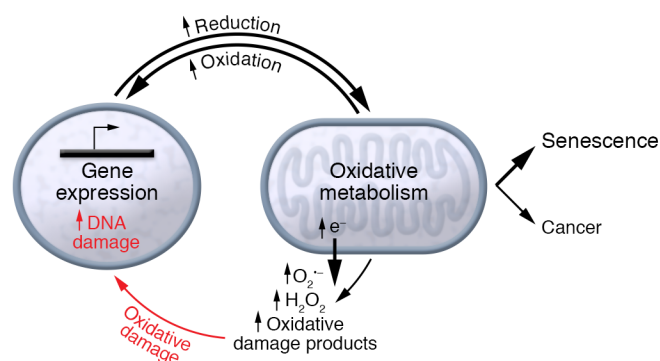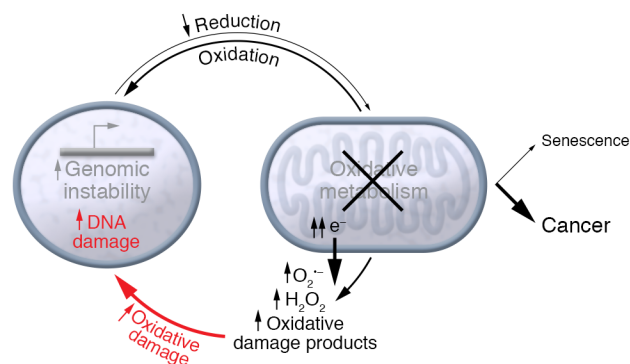
Linking metabolism and gene expression in cancer cells

In normally functioning cells, oxidative metabolism and gene expression are tightly coupled via the nonequilibrium steady-state fluxes of reactive metabolic by-products and electron carriers (i.e., NADH and $FADH_2$). Thus, in healthy cells, the flow of electrons

A Relationship between metabolism and gene expression

B Normal tissue damage

C Tumor metabolism

Figure 1. The horse and cart model describes the relationship between mitochondrial metabolism, signal transduction, and gene expression in mammalian biology, as well as in degenerative diseases associated with aging and cancer. (**A**) In healthy mammalian cells, the essential redox metabolic process occurring in the mitochondria and cytosol can be considered as the horse, and its related gene expression can be considered as the cart. In normal cells, oxidative metabolism and gene expression are tightly coupled via the nonequilibrium steady-state fluxes of reactive metabolic by-products and leaking electrons (e^-) carriers, such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). The level of DNA damage that occurs in a healthy cell is partially mitigated by ongoing DNA repair processes. (**B**) When the nonequilibrium steady state is disrupted, ROS and reactive oxidative by-products produced by oxidative metabolism (the horse) can increase oxidative damage in the genome, which will lead to the gradual deterioration of gene expression (the cart). Accumulation of DNA damage leads to cellular senescence or cancer. (**C**) The deregulated oxidative metabolism in cancer cells produces genomic instability that will eventually drag the cart off the cliff, so to speak, to the valley of death.

processes necessary for maintaining living systems can be considered as the horse that is tethered to the nuclear gene expression pathways by redox-sensitive signaling (Figure 1A). Thus, it is proposed that there is a mechanistic relationship between glucose and O_2 metabolism based on the ability to extract electrons from nutrient sources, via oxidation reactions, to generate ATP and reducing equivalents for biosynthesis while consuming O_2 to dispose of the electrons onto water molecules. These essential interconnected cellular metabolic processes are coordinately linked by the flow of ATP, reducing equivalents, and reactive by-products of oxidative metabolism (i.e., $O_2^{\cdot-}$, H_2O_2, organic hydroperoxides, etc.) through redox-sensitive signaling pathways to direct gene expression to control normal cellular functions that are closely matched to metabolic capabilities in the cell.

The horse and cart relationship can ultimately lead to a cellular environment that is permissive to gradual deterioration of multiple cellular processes, potentially due to stoichiometric mismatches between protein expression and metabolic machinery that impair efficient energy metabolism, as well as steadily increasing levels of reactive oxygen and nitrogen species. The resulting increasing inefficiency in oxidative metabolism and increasing steady-state levels of reactive species can lead to the exponential deterioration of the genome during aging, leading to senescence and death (Figure 1B). These age-related changes in reactive species and damage products are also accelerated by radiation and chemotherapy and can thus be targeted to inhibit normal tissue injury/regeneration during cancer therapy. In addition, Viale, Draetta, and colleagues showed that targeting mitochondrial respiration could induce the killing of oncogene ablation-resistant pancreatic cancer cells (Table 1), further suggesting the potential importance of targeting cancer cell metabolism in future therapeutic strategies (20).

In the case of carcinogenesis, we hypothesize that the emergence of a cellular and metabolic environment permissive for malignant transformation is due to the age-related loss of the horse and cart nonequilibrium steady state, leading to immortalization (possibly involving the induction of telomerase) as an adaptive response to the stress caused by oxidative damage to the genome. When the stoichiometry of ETC assembly is altered and creates mismatches, or the O_2 supply is altered, or the activity of

from energy-producing reactions should influence gene expression pathways that govern critical cellular processes to maintain a balance between normal cellular functions and differentiation. However, when this nonequilibrium steady state is disrupted, the reactive by-products of oxidative metabolism, such as $O_2^{\cdot-}$/H_2O_2, as well as reactive oxidative by-products (i.e., aldehydes and organic hydroperoxides), can cause covalent changes in the genome, leading to the gradual deterioration of metabolic gene expression pathways if unrepaired or misrepaired.

The horse (metabolism) and cart (gene expression) model provides a framework for understanding the fundamental relationship between cellular and mitochondrial metabolism, signal transduction, and gene expression in mammalian biology, as well as degenerative diseases associated with aging and cancer. In this regard, the essential mitochondrial and cytosolic redox metabolic

Table 1. Mutated mitochondrial metabolic genes and altered metabolites

Mutated gene (or altered metabolite)	Mitochondrial function affected	References
ND1	ETC complex I	130–140
ND2	ETC complex I	135, 137–139, 141
ND3	ETC complex I	137–139
ND4	ETC complex I	130, 135–139
ND4L	ETC complex I	131, 135, 137–139
ND5	ETC complex I	130, 131, 137–139, 142
ND6	ETC complex I	135, 137–139
CYTB	ETC complex III	131, 135, 137–139, 143
COXI[A]	ETC complex IV	135, 137–139, 143
COXII[A]	ETC complex IV	137–139, 142–144
COXIII	ETC complex IV	131, 135, 137–139
ATPase 6	ETC complex V	139, 141, 142
ATPase 8	ETC complex V	139, 143
Actionase 2	TCA cycle	54, 145–147
IDH[A]	TCA cycle	51–60
SDH[A]	TCA cycle	54, 62, 64–66
FH[A]	TCA cycle	54, 148–150
CS[A]	Oncometabolites	54, 78, 151
2-HG[A]	Oncometabolites	52, 54, 58, 152
MnSOD	Redox metabolism	153–155

[A]In clinical trial.

ETC proteins is altered, then the residence time of electrons on sites that can mediate one-electron reductions of O_2 increases, leading to increases in steady-state levels of O_2^{-} and H_2O_2 (21, 22). Likewise, when ADP is high or mitochondrial membrane potential is dissipated (by proton ionophores or uncoupling proteins), electron flows to cytochrome oxidase increase in an attempt to reestablish membrane potential, leading to increased four-electron reductions of O_2 to form H_2O. Finally, electron flows through ETC chains can be increased or reduced by shunting of carbon flux toward or away from the TCA cycle, respectively.

Given the complexity and multitude of proteins involved in regulating all of the aforementioned processes, it is logical to hypothesize that in fully transformed cancer cells, where mutation frequencies are estimated to be 200 times greater than in normal cells, oxidative metabolism becomes increasingly dysregulated relative to normal cells, contributing to continued progression to more aggressive malignant phenotypes. Once immortalization and malignant transformation occur, the living system has adapted to the dysfunctional relationship between oxidative metabolism, signal transduction, and gene expression; however, the transformed cells exhibit significant genomic instability, lose the ability to accomplish normal differentiation, lose control of cell proliferation, and continue to progress to malignancy. Moreover, the natural extension of this hypothesis is that continued targeting of only the malignant cell's dysfunctional gene expression, rather than also its aberrant metabolism (Figure 1C), significantly limits the potential ability to selectively kill cancer cells. Given this theoretical construct, we will discuss a model for targeting the hubs of disrupted normal oxidative metabolism in cancer cells that may

lead to new combined-modality approaches to improve the outcome of patients diagnosed with cancer while preserving normal tissue function.

NADPH in mitochondrial metabolism and tumor cell resistance

It is established that cancer cells exhibit disrupted metabolic pathways and processes that can subsequently lead to unregulated cell growth and a tumor-permissive phenotype. For instance, even in the presence of sufficient oxygen, cancer cells depend on increases in glycolysis for their energy production, which is referred to as either "aerobic glycolysis" or the "Warburg effect" (23, 24). Switching of metabolism to glycolysis can be a normal event after heavy exercise leading to the accumulation of lactate from anaerobic glycolysis. However, increasing glycolytic metabolism under oxidative stress also stimulates the regeneration of NADPH from $NADP^+$ in the pentose cycle, formation of ribose sugars necessary for purine and pyrimidine synthesis/DNA repair, and formation of pyruvate, which can directly scavenge H_2O_2, as well as feed TCA cycle intermediates, such as isocitrate and malate, which are also capable of regenerating NADPH (21, 22, 25–27).

NADPH serves as a source of reducing equivalents for the glutathione system, consisting of glutathione (GSH), glutathione disulfide (GSSG), glutathione peroxidases (GPx), and glutathione reductase (GR), and the thioredoxin system, consisting of thioredoxin ($Trx_{[SH]2}$), thioredoxin disulfide (Trx_{S-S}), peroxiredoxins (Prx), and thioredoxin reductase (TrxR). The glutathione and thioredoxin systems participate in the detoxification of H_2O_2 and organic hydroperoxides (ROOH) (Figure 2). $NADP^+$ can be reduced to NADPH by a variety of dehydrogenases, including glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of the pentose phosphate pathway; malic enzyme and isocitrate dehydrogenase (IDH) of the TCA cycle; and glutamate dehydrogenase (28). In addition to direct reduction by $NADP^+$-dependent dehydrogenases, the contribution of fatty acid oxidation in maintenance of NADPH and GSH levels is emerging in cancer biology (29, 30).

Recently, the contribution of the mitochondrial folate cycle enzymes mitochondrial methylene tetrahydrofolate dehydrogenase (MTHFD2) and aldehyde dehydrogenase-1-L2 (ALDH1L2) to NADPH production in cancer cells has become appreciated. MTHFD2, which is overexpressed in cancers (31), exhibits dual redox cofactor specificity and genetic inhibition of MTHFD2, or the cytosolic isoform (MTHFD1), decreasing NADPH/$NADP^+$ and GSH/GSSG ratios (32, 33). Further, NADPH produced by ALDH1L2 confers resistance to oxidative stress required by melanoma metastases (34). These findings are confirmed by studies demonstrating the reliance of cancer cells on exogenous serine and glycine, two amino acids that drive the folate cycle to maintain NADPH and GSH (34, 35). In addition to $NADP^+$-dependent dehydrogenases, NAD^+ kinase (NADK) has also been shown to exhibit control of NADPH levels in human cells, as phosphorylation of NAD^+ by NADK is the sole source of $NADP^+$ (36).

NAD^+ metabolism may also explain age-related increases in cancer incidence. For example, an age-dependent increase in CD38 activity is reflected by a decline in NAD^+, and this can be prevented by genetic or pharmacological CD38 inhibition (37, 38).
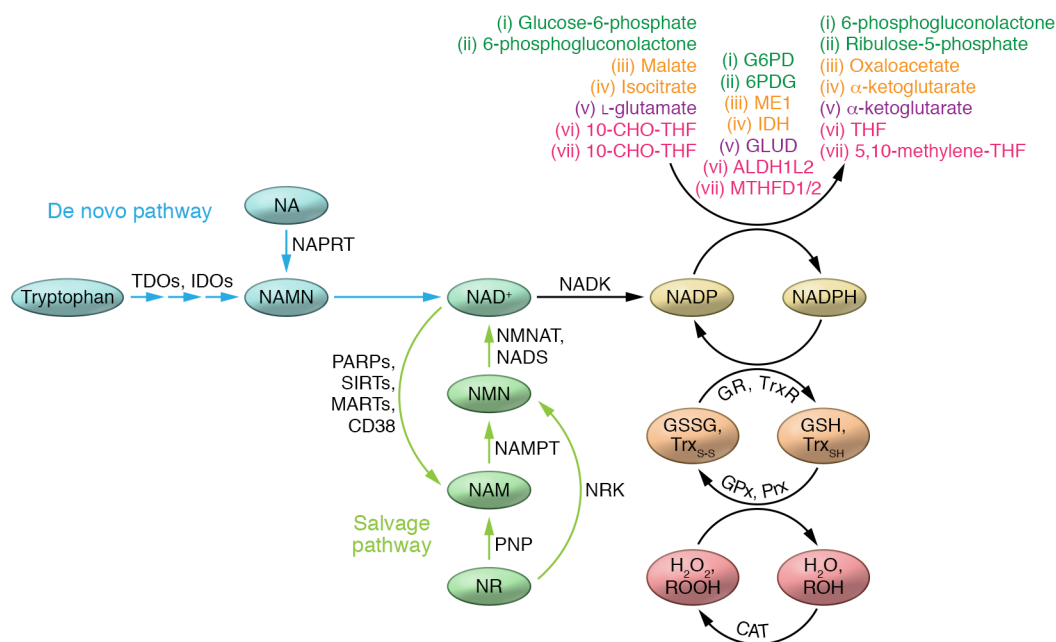
Figure 2. Interface of NAD synthesis and hydroperoxide metabolism. In the de novo synthesis pathway, L-tryptophan is converted to nicotinic acid mononucleotide (NAMN) in a series of eight steps. The initial, rate-limiting steps are catalyzed by tryptophan 2,3-dioxygenases (TDOs) and indolamine 2,3-dioxygenases (IDOs). The salvage pathway arm, controlled by the enzyme NAPRT, enters the pathway at this stage. NAMN is then enzymatically converted to NAD^+ by nicotinamide mononucleotide adenylyltransferase (NMNAT) and NADS (nicotinamide adenine dinucleotide sythetase). Nicotinamide (NAM) is an NAD^+ precursor generated by deacetylation and ADP-ribosylation reactions catalyzed by sirtuins (SIRTs), poly(ADP-ribose) polymerases (PARPs), mono-ADP ribosyltransferases (MARTs), and ADP-ribosyl cyclases such as CD38. NAM can also be synthesized by NR kinases (NRKs), which catalyze the phosphorylation of nicotinamide riboside (NR). Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the regeneration of nicotinamide mononucleotide (NMN) from NAM. NMN is then enzymatically converted into NAD^+ by NMNAT. $NADP^+$ is synthesized from NAD^+ by NAD kinase (NADK). $NADP^+$ can be reduced to NADPH by a variety of dehydrogenases. NADPH serves as a source of reducing equivalents for the glutathione system, consisting of GSH, GSSG, GPx, and GR, and the thioredoxin system, consisting of Trx_{SH}, Trx_{S-S}, Prx, and TrxR. The glutathione and thioredoxin systems participate in the detoxification of H_2O_2 and organic hydroperoxides (ROOH). Enzymatic reactions are color-coded to their metabolic pathway of origin: dark green, pentose phosphate pathway; orange, TCA cycle; fuchsia, one-carbon metabolism. G6PD, glucose-6-phosphate dehydrogenase; 6PDG, 6-phosphogluconate dehydrogenase; ME1, malic enzyme 1; 10-CHO-THF, 10-Formyltetrahydrofolate; GLUD, glutamate dehydrogenase; THF, tetrahydrofolate; CAT, catalase.

Age-dependent decreases in NAD^+ also correlate with reduced sirtuin activity, poly(ADP-ribose) polymerase (PARP) activity, and decreased mitophagy accompanied by mitochondrial dysfunction, increased ROS production, and genotoxicity (39–42). The emergence of many of these age-dependent phenomena can be slowed by NAD^+ precursor supplementation in the form of nicotinamide riboside, nicotinamide mononucleotide, or caloric restriction, suggesting that decreased NAD^+ plays a causal role in the aging processes (43–46). Thus, during aging, decreases in cellular NAD^+ result in the breakdown of PARP and sirtuin-mediated communication between the mitochondria (horse) and nucleus (cart) and subsequent loss of metabolic and genomic integrity (47, 48) that may explain the correlation between aging, mitochondrial dysfunction, and cancer incidence (49, 50).

Isocitrate is converted to α-ketoglutarate by isocitrate dehydrogenase-2 (IDH2), a key rate-limiting TCA cycle enzyme that produces NADPH. Several studies have suggested that mutations in IDH2 play a role in carcinogenesis, specifically colon cancer, prostate carcinoma, acute lymphoblastic leukemia, and glioblastoma (Table 1). These mutations inactivate wild-type IDH enzymatic activity, leading to 2-hydroxyglutarate production (2-HG). Increased 2-HG can impact α-ketoglutarate–dependent dioxygenase activity and alter various biological functions in tumors

(51–55). In this regard, the IDH2 inhibitor enasidenib is FDA-approved for acute myeloid leukemia, establishing TCA proteins as molecular targets (55–60).

NAD in mitochondrial metabolism and tumor cell resistance

Nicotinamide adenine dinucleotide is a coenzyme in redox reactions and thus exists as an oxidized form (NAD^+), as well as a reduced form (NADH). NAD metabolism has recently emerged as an intriguing molecular target to modify mitochondrial oxidative metabolism (61). For example, it was shown that circadian rhythms influence mitochondrial metabolism through cellular pathways that direct NAD^+/NADH levels, as well as through the acetylation of key mitochondrial redox and detoxification proteins (61). Cells maintain significant concentrations of both NAD^+ and NADH with a high NAD^+/NADH ratio (~700 in the cytoplasm, 3–10 in the cell as a whole) that is favorable for oxidative reactions (2–4). In contrast, NADPH plays a major function as a reducing agent in anabolism, such as in fatty acid synthesis and photosynthesis, and thus the $NADP^+$/NADPH ratio is kept very low (about 0.005) (4, 5). $NADP^+$/NADPH, which exists at roughly one-tenth of the concentration of NAD^+/NADH, is also a major chemical redox balance responder in cells. A Swiss-Prot database (accessed May 2018 at

UniProt, www.uniprot.org) showed 20,349 entries for human proteins and 459 proteins that utilize NADs (NAD+, NADH, NADP+, and NADPH combined), which is 2.3% of all human proteins.

Succinate dehydrogenase (SDH) is one of the proteins that utilize NADs and is involved in two essential cellular metabolic processes, the TCA cycle and the ETC. Mutations in the subunits of SDH (SDHA, SDHB, SDHC, and SDHD) in paraganglioma and gastrointestinal stromal tumor, renal carcinoma, and pheochromocytoma lead to loss of enzymatic function, increased ROS production (54, 62, 63), and accumulation of succinate (Table 1). ROS, as well as succinate, can independently or synergistically lead to the induction of a hypoxic response. In addition, accumulated succinate may inhibit α-ketoglutarate–dependent dioxygenases and prolyl hydroxylase domain protein 3–mediated (PHD3-mediated) apoptosis. In this regard, certain drugs, like SGI-110 (guadecitabine) and temozolomide, have been used in clinical trials for gastrointestinal stromal cancer, paraganglioma, and renal cell carcinoma (64–66).

Mitochondrial compartmentalization of NAD+

Although NADH is free to enter the intermembrane space through the outer mitochondrial membrane, it is not permeable into the inner mitochondrial membrane (30). Instead, the reducing equivalent of NADH is transported into the mitochondria via either the malate-aspartate shuttle or the glycerol-3-phosphate shuttle of the inner mitochondrial membrane (31). In addition, the different NAD+ compartmental pools (i.e., nucleus, cytoplasm, mitochondria) can behave independently. As such, the maintenance of NAD+ levels in each compartment is dependent on the activities of transportation and salvaging of the nicotinamide (NAM) produced by NAD+-consuming enzymes via nicotinamide phosphoribosyltransferase (NAMPT) and nicotinamide mononucleotide adenylyltransferase (NMNAT) activities (15). In fact, cancer cells appear to rely much more heavily on NAMPT than normal cells, and this pathway has been suggested as a potential target for radio-chemo-sensitization (67–70). Therefore, each NAD+ pool is tailored to compartment-specific metabolic needs. For example, nuclear-localized NAD+ was recently reported to regulate adipocyte differentiation (32).

Sirtuins (SIRTs) are a major target of NAD+ signaling and direct, at least in some part, the acetylation status of mitochondrial proteins. In this regard, the total intracellular concentration of NAD+ in mammals is estimated between approximately 200 and 500 μM (6, 33–35), and the binding affinity (K_m) of NAD+ to SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, and SIRT6 is reported as 94–96 μM (36, 37), 83 μM (38), 880 μM (39), 35 μM (40), 980 μM (41), and 26 μM (42), respectively. Based on these data, it seems reasonable to propose that sirtuins can be classified into two categories: (a) SIRT2, SIRT4, and SIRT6, whose activity is unlikely to be rate-limited by NAD+, as NAD+ availability is much higher than their K_m; and (b) SIRT1, SIRT3, and SIRT5, whose K_m for NAD+ falls within the physiological NAD+ range. As such, it seems important to note that SIRT1 localizes in the nucleus, and NAD+ levels in the nucleus are below 100 μM, while NAD+ concentrations in the mitochondria range up to the millimolar order. Thus, owing to their K_m values, NAD+ could operate SIRT3 and SIRT5 as NAD+ sensors. It is therefore proposed that the different concentrations of NAD+ in the various subcellular compartments may direct a specific cellular process downstream of the sirtuin proteins, and that mitochondrial sirtuins may be a potential molecular target for cancer therapeutics (71–74).

In this regard, it appears that slightly more than 65% of all mitochondrial proteins are constantly acetylated nonenzymatically because of the high concentration of acetyl-CoA and high pH (pH ~8.0) within the mitochondrial compartment (49–51). Therefore, it is proposed that the mitochondrial deacetylases play primary roles in regulating the mitochondrial acetylome network (39, 52–55). In addition, it seems reasonable to suggest that SIRT3, the primary mitochondrial deacetylase, also functions to deacetylate many mitochondrial proteins, e.g., in response to calorie restriction, fasting, and other conditions in which nutrient availability is limited (49, 50). SIRT3 plays critical roles in mitochondrial functions, such as repair, respiration, and dynamics. While SIRT3 directs the acetylation and enzymatic activity of a number of proteins involved in energy generation, it also regulates several key detoxification proteins that regulate cellular ROS. SIRT3 and its downstream proteins may be important molecular biomarkers and/or target proteins that alter mitochondrial ROS, leading to a cellular phenotype permissive for tumor cell cytotoxicity.

Mitochondrial stress responses in carcinogenesis

One of the best-described mechanisms for mitochondrial communication with other cellular compartments is through the release of ROS, and mitochondrial oxidants function in numerous signaling pathways (75–77). The mitochondrial respiratory chain is responsible for the production of ROS (19, 25, 78), and in normal cells, steady-state levels of ROS, via either production or detoxification, are tightly controlled and can act as secondary messengers to direct normal cellular functions (79). However, in stressed mitochondria, ROS can exist at nonphysiological levels that, in a specifically cellular context, may be harmful by damaging proteins, lipids, and DNA, thus contributing to mitochondrial dysfunction and carcinogenesis (42, 45–48).

SIRT3 has been demonstrated as a tumor suppressor that inhibits mitochondrial ROS production, at least in some significant part, through the activation of downstream deacetylation targets including SOD2, IDH2, and FoxO3a (52, 53, 59–62). Loss of SIRT3 also stabilizes hypoxia-inducible factor-1α (HIF-1α) as a result of increased ROS levels, directing metabolic pathways toward aerobic glycolysis to support tumor growth (52, 59). SIRT3 also regulates the malate-aspartate shuttle (63), which is critical to sustain a high rate of glycolysis in tumor cells, and mitochondrial glutamate oxaloacetate transaminase, both of these enzymes are SIRT3 targets and are the key components of the glycolysis shuttle. Further supporting its tumor suppressor role, expression of SIRT3 is downregulated in breast cancer, colon carcinoma, osteosarcoma, and hepatocellular carcinoma, and mice lacking *Sirt3* spontaneously develop estrogen receptor–positive, high–Ki-67, poorly differentiated mammary tumors (53, 59, 64–66). Based on these results, it seems reasonable to propose that SIRT3 is positioned at the interface between NAD+/NADH biology and cellular ROS levels/detoxification enzymes, and as such, SIRT3 may be a potential target for the development of novel anticancer agents in these tumor types.

Targeting mitochondrial protein import/transport in anticancer interventions

The majority of mitochondrial precursor proteins encoded in the nucleus contain an N-terminal matrix-targeting sequence (MTS) to facilitate localization to the mitochondrial matrix. The MTS-containing proteins are first recognized by the translocase of the mitochondrial outer membrane complex (TOM complex) and then by the translocase of the inner membrane 23 complex (TIM23 complex) in the inner membrane (67). Upon translocation into the mitochondria, MTS is cleaved, and these proteins undergo chaperone-assisted folding and transport to specific locations (68). Protein trafficking requires ATP and the proton gradient generated by the respiratory chain, and thus would be disturbed by oxidative phosphorylation (OXPHOS) defects. Importantly, protein trafficking through the TIM23 complex is sensitive to multiple stressors and is inhibited by elevated ROS, mitonuclear protein imbalance, the status of the matrix-localized chaperone mtHSP70 (also known as HSPA9), and unfolded proteins as described below (69–71). In other words, TIM23-mediated protein trafficking is capable of pushing the cart and needs to be compensated by the horse.

The mitochondrial unfolded protein response (UPRmt) is a transcriptional program regulated by defined transcription factors, which are activated by mitochondrial perturbation/dysfunction through mitochondrial-nuclear communication. It is an adaptive stress response that functions to resolve the accumulation of unfolded proteins within mitochondria, which leads to oxidative stress, similar to its effect within other cellular compartments. Although the functions of many UPRmt-induced genes are still unknown, it is well established that the UPRmt is activated if mitochondrial function declines to promote repair and recovery.

Mitochondria contain multiple copies of a small circular genome (mtDNA) that encodes 13 OXPHOS proteins, as well as transfer RNAs and ribosomal RNAs required for their synthesis in the mitochondrial matrix space (72). The rest of the approximately 1,200 mitochondrial proteins are encoded by nuclear genome, translated in cytoplasm, and subsequently imported into mitochondria (73). Therefore, the disruption of stoichiometric balance between components of OXPHOS complexes I, III, IV, and V, including proteins encoded by both the mitochondrial and nuclear genomes, triggers UPRmt activation. In *Caenorhabditis elegans*, the activating transcription factor associated with stress (ATFS-1) is one of the transcription factors required for UPRmt, and contains an MTS in addition to a nuclear localization signal (NLS) (74). In cells with healthy mitochondria, the MTS prevails, and ATFS-1 is imported into mitochondria and degraded by the matrix-localized protease LON. When mitochondria are damaged, the NLS directs ATFS-1 to the nucleus to activate transcription (74), and thus mitochondrial protein import is a key UPRmt regulatory mechanism. ATFS-1 also temporarily reduces OXPHOS gene transcription in both the nucleus and the mitochondria with increasing transcription of glycolysis components (74, 75), which likely allows cells to generate ATP in the cytosol to ensure survival and mitochondria recovery. Thus, the UPRmt and ROS appear to be closely connected in the mitochondria, and it seems reasonable to suggest that agents targeting this pathway, as well as the pathways discussed above, could be novel therapeutic interventions in the treatment of cancer patients.

Aberrant mitochondrial oxidative metabolism in redox-sensitive cancer cells

Glutathione (GSH) and the related enzymes, glutathione peroxidases (GPx1 and GPx4), glutaredoxin 2 (80), and glutathione reductase (GR), make up the major components of mitochondrial thiols that maintain redox reactions and mitochondrial metabolic homeostasis (81). In addition, thioredoxin-2 (Trx2), peroxiredoxin-3 (Prx3), and peroxiredoxin-5 (Prx5) also play functional roles in mitochondrial thiol metabolism (82, 83). GSH in mammalian cells participates in the detoxification processes by efficiently removing various hydroperoxides through its role as a cofactor for glutathione peroxidase, glutathione-*S*-transferases, and sulfiredoxins (84, 85). This mitochondrial thiol antioxidant system supports and provides electron sources for both the thioredoxin and glutaredoxin systems via NADPH, which is a major product of nicotinamide nucleotide transhydrogenase, glucose 6-phosphate dehydrogenase, and IDH2 (86). Folate metabolism is also strongly linked to thiol metabolism through the regulation of NADPH, formate, purine biosynthesis, and GSH metabolism (87–91), providing many new points of cancer cell–specific therapeutic intervention.

GSH also plays a role in cellular viability and redox balance by reducing peroxides, acting as a nucleophile as it shuffles target proteins between oxidized and reduced states, as well as the detoxifying lipid peroxidation–derived aldehydes (80, 91–95). Because mitochondrial GSSG cannot be exported, the dynamic ratio between GSH and GSSG could provide a controllable environment for disulfide bond formation during the folding of nascent proteins (95). Thus, it seems reasonable to propose that the dynamic ratio between GSH and GSSG, as well as the ratio between $Trx_{[SH]_2}$ and Trx_{S-S}, which is disrupted in cancer cells, directs NADH/NADPH metabolism, folate metabolism, and ETCs.

The mitochondrial thiol antioxidants constitute an integrated regulatory system that exerts functions in a wide range of biological activities in mammalian cells, including maintenance of steady-state levels of hydrogen peroxide (H_2O_2) and organic hydroperoxides (ROOH), which have an important role in maintaining mitochondrial and cellular redox homeostasis. Disruption of mitochondrial thiol antioxidant systems leads to accumulation of oxidative damage to lipids, proteins, and DNA, as well as proper assembly and function of ETCs. This oxidative damage contributes to radio-chemo-sensitization due to increased levels of $O_2^{·-}$ that liberate redox-active metals from ferritin (Fe^{2+}), and reduce Fe^{3+} to Fe^{2+}, during redox cycling of pharmacological levels of ascorbate to selectively generate more ROS, as well as hydroxyl radical (·OH) through the Fenton reaction (96, 97). In addition, mitochondrial ETC-generated superoxide ($O_2^{·-}$) is converted by manganese superoxide dismutase (MnSOD) to H_2O_2, and this reaction is thought to be tumor suppressive (98). Reactive nitrogen species (RNS) can also be generated in the form of nitric oxide (·NO), which can react with $O_2^{·-}$ (99, 100) to form peroxynitrite, which plays an important role in thiol-sensing pathways as well as anticancer therapy cytotoxicity. In contrast, the subsequent accumulation of aberrant levels of ROS or RNS can lead to oxidative and nitrosative damage, leading to genomic instability, protein and lipid oxidation, and altered mitochondrial metabolism, all of which lead to altered cell survival, growth, and proliferation (101, 102).

Among the different ROS generated in mitochondrial oxidative and respiratory metabolism, H_2O_2 is considered as one of the most

critical redox signaling molecules and one of the ROS most relevant to cancer therapy (96). The levels of mitochondrial H_2O_2 are determined by a wide range of processes, including MnSOD, which is the key modulator for controlling O_2^{-} redox balance (98, 103, 104). The mitochondrial thiol system is crucial to maintain the redox balance between production and elimination of H_2O_2 and other organic hydroperoxides. In addition, many studies have shown that the disruption of the correct physiological balance of mitochondrial H_2O_2/ROS homeostasis is a driving factor, at least in some part, in many diseases, such as carcinogenesis/tumorigenesis (19, 74, 105, 106).

It is an established paradigm in cancer research that thiol pathways, whose function is altered in tumor cells, are also potential molecular targets for new therapeutic interventions (98, 107–109). Moreover, compared with normal healthy cells, tumor cells exhibit an increased steady-state level of ROS, including H_2O_2, due to the altered mitochondrial metabolic processes and uncontrolled proliferation (22, 96, 104, 110). Although it was previously thought that ROS are always cytotoxic in both normal cells and cancer cells, it is not that simple. There is an essential role for O_2^{-} in normal signaling processes. Moreover, a certain amount of ROS (H_2O_2 in particular) stimulates cancer cell proliferation through modifications in the signaling pathways (111, 112). This idea fits comfortably into the cart and horse model, whereby the H_2O_2 produced by the "horse" signals alterations in gene expression through the redox-sensitive thiol signaling networks (Figure 1).

In regard to H_2O_2 signaling, studies have demonstrated that H_2O_2 in tumor cells stimulates the phosphorylation of PI3K/AKT, activates the HIF-1α pathway (77, 113, 114), and alters the tumor microenvironment that promotes angiogenesis through EGF activation, all of which promote a tumor-permissive phenotype (112, 113). Because of the close link between ROS, thiol redox signaling, and cellular processes, thiol antioxidant machinery plays an important role in regulating the redox balance and participates in cell proliferation and survival, cellular metabolism, and genomic integrity. H_2O_2 has also been shown to reversibly oxidize critical cysteine thiol groups of phosphatases, and H_2O_2 can hyperactivate PI3K/AKT/mTOR by oxidizing and inactivating the phosphatases PTEN and PTP1B, which are negative regulators of PI3K/AKT signaling (115, 116). Moreover, H_2O_2 could also oxidize prolyl hydroxylase domain protein 2 (PHD2), which can lead to the oncogenic stabilization of HIF-1α protein and promote oncogenic transformation.

In this regard, targeting of deregulated redox pathways in cancer cells is becoming increasingly important in experimental therapeutics. Specifically, agents such as metformin or phenformin appear to inhibit the proliferation and growth of certain types of cancer (117, 118). Several studies have shown that metformin could inhibit ETC complex I or the mTOR pathway by activating AMPK, and further decrease activation of IGF-1/insulin receptor, AKT, and ERK signaling, which could eventually lead to growth inhibition and/or apoptotic death. Because of the promising effects of metformin on AMPK-mediated redox-sensitive pathways, there are many trials assessing the usefulness of metformin in cancer treatment and cancer prevention (118–121).

In addition, in tumor biology, many studies have also suggested that mitochondrial thiols are involved in tumorigenesis, tumor progression, metastasis of cancer cells, and tumor therapy resistance (122–124). Recent studies also suggested that H_2O_2 could

rely on thiol antioxidant systems to sense and transduce the redox signals (125). In mammalian cells, thiol antioxidant enzymes like Prx or GPx can receive H_2O_2 oxidation and transfer the oxidation to the target protein. For example, mitochondrial Prx has been shown to be reversibly hyperoxidized and inactivated by H_2O_2 to allow for localized H_2O_2 accumulation for target protein oxidation (126, 127). In addition, it has been suggested that GSH or reduced Trx recycles the oxidized scavenger back to its reduced state and transmits the signal to the target protein, and H_2O_2 oxidation of the scavenging enzyme may result in target protein dissociation and activation (94, 122). These critical thiol-based redox-sensitive signaling pathways, coupled with the increased steady-state levels of mitochondrial ROS in cancer cells, provide a clear biochemical rationale for a combined modality of cancer cell targets for selectively sensitizing cancers to cytotoxic therapeutic agents, while sparing normal tissues from the devastating effects of chemotherapy and radiation.

Conclusions

The disruption of normal mitochondrial physiology is a hallmark of cancer (128, 129), and with each passing year, our understanding of the link between aberrant metabolism and carcinogenesis grows. In this regard, the horse and cart model exhibits the relationship among the mechanisms by which tumor cells establish aberrant regulation of cellular and mitochondrial metabolism, signal transduction, and gene expression in cancer biology. Specifically, it has been proposed that oxidative metabolism (the horse) and gene expression (the cart) are normally tightly coupled through non-equilibrium steady-state fluxes of reactive metabolic by-products, electron flow from reactions governing energy production, and the cellular pathways governing growth and development. In addition, coordinated energy production, and the generation of reducing equivalents required for normal cellular physiology, deteriorate as an exponential function of aging and during progression to malignancy. Thus, this model predicts that metabolic oxidation reactions, when disrupted, lead to the formation of ROS and oxidative products detrimental to normal cellular processes and a damage-permissive phenotype of diseases associated with aging and cancer that can be targeted to improve human health.

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