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Pro- and antitumor effects of mitochondrial reactive oxygen species

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Abstract
In cancer, mitochondrial functions are commonly altered. Directly involved in metabolic reprogramming, mitochondrial plasticity confers to cancer cells a high degree of adaptability to a wide range of stresses and to the harsh tumor microenvironment. Lack of nutrients or oxygen caused by altered perfusion, metabolic needs of proliferating cells, co-option of the microenvironment, control of the immune system, cell migration and metastasis, and evasion of exogenous stress (e.g., chemotherapy) are all, at least in part, influenced by mitochondria. Mitochondria are undoubtedly one of the key contributors to cancer development and progression. Understanding their protumoral (dys)functions may pave the way to therapeutic strategies capable of turning them into innocent entities. Here, we will focus on the production and detoxification of mitochondrial reactive oxygen species (mtROS), on their impact on tumorigenesis (genetic, prosurvival, and microenvironmental effects and their involvement in autophagy), and on tumor metastasis. We will also summarize the latest therapeutic approaches involving mtROS.

Keywords Cancer · Mitochondria · Mitochondrial reactive oxygen species (mtROS) · Antioxidants · Pro-oxidants · mitoQ

1 Introduction

Mitochondria are essential organelles for cell viability. They fulfill multiple functions: (*i*) they host important metabolic pathways for bioenergetics (tricarboxylic acid [TCA] cycle, fatty acid oxidation, and electron transport chain [ETC]) and biosynthesis (TCA cycle, heme and Fe/S cluster synthesis), (*ii*) their intermembrane space (IMS) contains proteins necessary to regulate the intrinsic apoptotic pathway, (*iii*) they store and release Ca^{2+} for signaling, and (*iv*) they are one of the most important production sites of reactive oxygen species (ROS) in cells [1, 2]. Mitochondria also have a central role in adaptation to different kind of microenvironmental stresses through a process generally known as mitohormesis. In the case of acidosis, a common condition in the tumor microenvironment, specific mitohormetic responses of cancer cells switch glycolytic metabolism towards oxidative phosphorylation (OXPHOS) fueled by glutamine, fatty acids, and lactate which decreases extracellular acidification [3, 4], increase fatty acid oxidation in parallel to fatty acid synthesis [5], and increase mitochondrial ROS (mtROS) production, which promotes invasiveness/escape from acidity [6, 7]. Here, we will detail how mitochondrial functions may affect tumor development.

Special issue: *Acidosis Cancer* - Robert Gillies, Guest Editor

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2 Mitochondrial ROS generation

2.1 ROS chemistry

Superoxide ($O_2^{\bullet-}$) is a free radical produced by the single electron reduction of O_2. M. Murphy [8] termed it "the proximal ROS" because it is the first ROS directly produced from O_2 and the precursor of all other ROS (**Reactions 1–3**): spontaneous and superoxide dismutase (SOD)-dependent $O_2^{\bullet-}$ dismutation generates hydrogen peroxide (H_2O_2), which itself can undergo the Fenton reaction to generate the hydroxyl radical ($OH^{\bullet}$) in the presence of transition metals, most commonly Fe^{2+}. The oxidative reactivity of the oxygen atom with cell components (nucleic acids, lipids, proteins) progressively

increases during this reaction cascade and culminates with $OH^{\bullet}$ that is extremely unstable and immediately reacts at its production site [2]. Here, we will focus on $O_2^{\bullet-}$ as a precursor of signaling agent H_2O_2, which has an intermediary reactivity. Of note, $O_2^{\bullet-}$ can react with nitric oxide ($NO^{\bullet}$) to generate peroxynitrite ($ONOO^-$) (**Reaction 4**), a reactive nitrogen species (RNS) that will not be discussed here [8].

$$O_2 + e^- \rightarrow O_2^{\bullet-}$$
$$2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2$$
$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+} \text{(Fenton reaction)}$$

Reactions 1–3: the ROS cascade

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$$

Reaction 4: peroxynitrite formation

2.2 Mitochondrial ROS generation and diffusion

Studies on isolated mitochondria showed that the ETC is the major $O_2^{\bullet-}$ production site in these organelles (Fig. 1) [8]. The ETC comprises four complexes regulating electron transfer from reduced cofactors NADH and $FADH_2$ to the final electron acceptor O_2 in order to pump H^+ from the mitochondrial matrix to the IMS through ETC Complexes I, III, and IV. NADH is oxidized at Complex I (NADH dehydrogenase), and its electrons are successively transferred from Complex I to ubiquinone, Complex III (ubiquinone-cytochrome *c* reductase), cytochrome *c*, Complex IV (cytochrome *c* oxidase), and O_2. $FADH_2$ provides electrons to Complex II (succinate dehydrogenase [SDH], which is also a TCA cycle enzyme) that then shuttle from Complex II to ubiquinone, Complex III, cytochrome c, and Complex IV and ultimately react with O_2. ATP synthase, also termed Complex V, uses the energy from the H^+ gradient to phosphorylate ADP in ATP when H^+, following their concentration gradient, returns from the IMS to the mitochondrial matrix *via* the enzyme (**Reaction 5–6**). The full process is termed OXPHOS.

$$NADH + H^+ + {}^1/_2\ O_2 + 3\ ADP + 3\ Pi \rightarrow NAD^+ + 3\ ATP + 4\ H_2O$$

Reaction 5: OXPHOS with NADH

$$FADH_2 + {}^1/_2\ O_2 + 2\ ADP + 2\ Pi \rightarrow FAD + 2\ ATP + 3\ H_2O$$

Reaction 6: OXPHOS with $FADH_2$

Because they comprise flavin-containing prosthetic groups, Fe/S clusters, and ubiquinone-binding sites, ETC complexes, particularly Complexes I and III, can, under certain conditions, transfer a single electron to O_2 and generate $O_2^{\bullet-}$ [2]. In isolated mitochondria, four modes of $O_2^{\bullet-}$

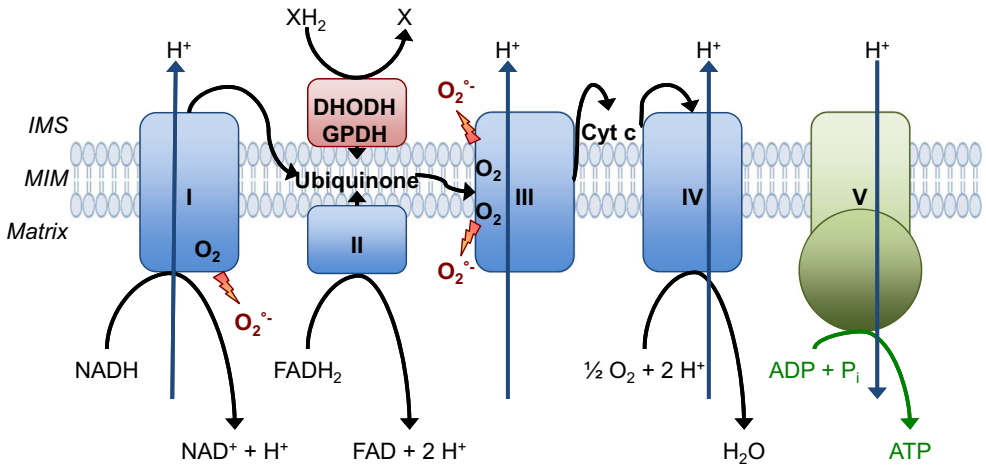

Fig. 1 The mitochondrial electron transport chain is a major site of reactive oxygen species production. The electron transport chain (ETC) is located on the mitochondrial inner membrane (MIM). Complexes I and II receive electrons from reduced cofactors NADH and $FADH_2$, ubiquinone then collects electrons from Complexes I and II, from dihydroorotate dehydrogenase (DHODH), and from glycerol-3-phosphate dehydrogenase (GPDH) [8]. Electrons are successively transferred from reduced ubiquinone to Complex III, cytochrome c (Cyt c), and Complex IV towards their final acceptor O_2. Complexes I, III, and IV use the energy released by electron transfer to pump H^+ from the mitochondrial matrix to the intermembrane space (IMS), thereby generating a transmembrane H^+ gradient to support ATP generation by Complex V. ROS generation occurs *via* single electron leakage under specific conditions: (i) at Complex I and at Complex III under physiological functions of the ETC, (ii) at Complex I when the NADH/NAD^+ ratio is high and ETC activity is low, (iii) at Complex I by reverse electron transfer when the pool of reduced ubiquinone and the transmembrane H^+ gradient are high, and (iv) at Complex III under hypoxic conditions [8]. Complex I releases $O_2^{\bullet-}$ in the stroma and Complex III in the IMS and in the stroma [2, 9, 10]. Despite Complex I and Complex III are considered to be the main sources of mtROS, other proteins, including Complex II, DHODH, GPDH, α-ketoglutarate dehydrogenase, and proline dehydrogenase, are additional sites of $O_2^{\bullet-}$ production [2, 8, 11]

production have been characterized so far [8]. First, electron leakage occurs physiologically at Complexes I and III under normal function of the ETC [8]. xIt is probably the most important, but also the least well-characterized, mode of $O_2^{\bullet-}$ production in mitochondria. In the particular case of extracellular acidosis, increased leakage could account for increased mtROS production as a mere consequence of increased OXPHOS activities [12]. Second, when the respiration rate is low and the NADH/NAD^+ ratio is high, Complex I produces $O_2^{\bullet-}$ at the NADH oxidation site [8, 11]. Third, when the H^+ gradient across the mitochondrial inner membrane (MIM) is steep and when reduced ubiquinone is abundant, electrons are transferred back from ubiquinone to Complex I (reverse electron transfer), which produces $O_2^{\bullet-}$ [2, 8]. Fourth, a low O_2 availability, hypoxia, induces a paradoxical increase in $O_2^{\bullet-}$ production by mitochondria at Complex III, which works as an O_2 sensor. Under hypoxia, through a yet poorly understood mechanism, an altered flux of electrons at sites Q_0 and Q_i of Complex III generates $O_2^{\bullet-}$ [2, 8, 9, 11, 13, 14].

Both the location of electron leakage relative to the MIM and the electrical field across the MIM are susceptible to orient electron leakage and $O_2^{\bullet-}$ production towards either side of the membrane [2, 9, 10]. Complex III releases $O_2^{\bullet-}$ at both sides of the MIM, whereas Complex I exclusively releases $O_2^{\bullet-}$ towards the mitochondrial matrix [8, 10]. Importantly, $O_2^{\bullet-}$ diffusion across mitochondrial membranes is facilitated by anion channels and the diffusion of cell-permeable H_2O_2 by aquaporins [2, 8]. Therefore, the site of ROS production does not determine their site of action in absolute terms, although a relative proximity of their target is required due to their relatively high chemical reactivity [9]. Nonetheless, ROS produced in mitochondria can reach the cytosol. The relative contribution of mitochondrially derived $O_2^{\bullet-}$ and H_2O_2 to cytosolic processes is difficult to assess. Chemical considerations nevertheless indicate that $O_2^{\bullet-}$ has a very short half-life in biological systems [2, 8, 15] and limited reactivity with cell components in comparison to H_2O_2 [16], suggesting that $O_2^{\bullet-}$ may be seen as a precursor and H_2O_2 as an effector in these processes.

In addition to ETC complexes, other mitochondrial enzymes comprising a flavin-containing prosthetic group, a Fe/S cluster, or binding to ubiquinone can release $O_2^{\bullet-}$ under particular redox conditions [2]. ROS production was indeed reported for TCA cycle enzymes α-ketoglutarate dehydrogenase [8], SDH/Complex II [8], proline dehydrogenase [11], pyruvate dehydrogenase [17], branched chain α-ketoacid dehydrogenase [17] and 2-oxoadipate dehydrogenase [18] in the mitochondrial matrix, and dihydroorotate dehydrogenase and glycerol-3-phosphate dehydrogenase in the IMS [8].

2.3 Antioxidant defenses

In cells, ROS production is normally counterbalanced by cellular antioxidant defense systems. In mitochondria, SODs are very efficient catalysts that convert $O_2^{\bullet-}$ into H_2O_2, although this reaction also occurs spontaneously [2, 8]. SOD1 is present in the cytosol and IMS and SOD2 in the mitochondrial matrix, whereas SOD3 is extracellular [2, 8, 9]. The SOD-produced H_2O_2 can subsequently be reduced to H_2O by peroxidases: catalase, glutathione peroxidases (GPxs, eight isoforms), and peroxiredoxins (Prxs, six isoforms) [2, 9]. Among them, catalase, GPx1, GPx4, Prx3, and Prx5 are present in the mitochondrial matrix [8, 19]. Importantly, GPx activity requires reduced glutathione (GSH) as an electron donor, and GSH regeneration from GSSG depends on the activity of NADPH-consuming glutathione reductase (one isoform, also present in the mitochondrial matrix) [2, 9, 19]. The regeneration of reduced Prxs occurs through oxidation of thioredoxins (two isoforms), themselves recycled by NADPH-dependent thioredoxin reductases (three isoforms) [2, 9], of which thioredoxin 2 and thioredoxin reductase 2 are expressed in the mitochondrial matrix [8, 19]. Thus, GSH synthesis (*via* cystine and cysteine uptake [20–22], glutamine metabolism and the serine pathway) and NADPH production (by the pentose phosphate pathway [PPP], the folate pathway, NADH/NADPH transhydrogenase, isocitrate dehydrogenases (IDHs) 1 and 2, and malic enzymes [ME] 1 and 3) actively support H_2O_2 detoxification [2, 23]. GSH transport from the cytosol to the mitochondrial matrix occurs *via* porin channels through the mitochondrial outer membrane and α-ketoglutarate and dicarboxylate transporters through the MIM [19]. NADPH can be produced *in situ* by NADH/NADPH transhydrogenase, IDH2, and ME3 [2, 8].

Interestingly, the transcription of several antioxidant enzymes depends on a redox-sensitive interaction between transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and its repressor kelch-like ECH-associated protein 1 (KEAP1) [2, 24]. In normal conditions, Nrf2 interacts with KEAP1 that prevents Nrf2 nuclear translocation, and Nrf2 is targeted to the proteasome for degradation by ubiquitin ligase cullin 3 [24]. In pro-oxidant conditions, specific cysteine residues of KEAP1 are oxidized, KEAP1 releases Nrf2, and Nrf2 translocates to the cell nucleus where it induces the transcription of genes encoding, e.g., GSH-synthetizing enzymes, GPx2, Prx1, Prx6, thioredoxin 1, and thioredoxin reductase 1 [24].

Altogether, net mtROS production depends on (*i*) the rate of ROS production, (*ii*) the site of ROS production, (*iii*) ROS transport, and (*iv*) the efficiency of ROS clearance by cellular antioxidant defense systems.

3 Mitochondrial ROS production in tumors

Because of their high reactivity, (mt)ROS oxidize proteins (particularly thiol residues), lipids, and nucleic acids, either behaving as damaging or as signaling species according to their nature, their amount, their location, and cell sensitivity.

For a long time, ROS in general and mtROS in particular have almost exclusively been considered as mediators of apoptosis, lipid peroxidation, and DNA damage. However, their roles as signaling agents between mitochondria and the cytosol are emerging in physiology (immunity, cell differentiation, autophagy, hypoxia, ageing [9]) and in pathology, including in cancer. Compared to normal cells, cancer cells often have higher levels of ROS in general and mtROS in particular, which can be explained by several factors: increased mitochondrial oxidative metabolism, mitochondrial DNA mutations, microenvironmental conditions, amplification loops, and saturated antioxidant defenses [9, 25, 26].

3.1 Mitochondrial oxidative metabolism and ROS production in tumors

Oncogenic phosphoinositide-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR), Myc, and hepatocyte growth factor receptor Met/Rac1 pathways are all associated with stimulation of mitochondrial metabolism and, consequently, ROS production [2, 11, 27–29]. Intuitively, because $O_2^{\bullet-}$ is a natural by-product of ETC activity, increased ETC flux and/or more mitochondria would also mean increased mtROS production upon adequate substrate and oxygen supply. However, this relationship is not necessarily straightforward, as illustrated by the dual role of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a master regulator of mitochondrial metabolism and mitochondrial biogenesis [9, 30]. Expression of PGC-1α increases during breast cancer progression [31, 32] but decreases during prostate cancer [33] and melanoma [34] development. In addition to the stimulation of mitochondrial functions, PGC-1α also induces the expression of SOD1, SOD2, catalase, GPx1, and PPP enzymes that all contribute to ROS detoxification [30, 35]. Moreover, differential transcriptional programs according to nutrient abundance and PGC-1α isoforms make more complex the evaluation of PGC-1α impact on mitochondrial biogenesis, oxidative metabolism, and mtROS levels [30, 36].

Conversely, ATPase inhibitory factor 1 (ATPIF1) overexpression, detected in several cancers types including breast, lung, and ovarian cancers, causes metabolic reprogramming from an oxidative metabolism to aerobic glycolysis [37, 38]. Accordingly, overexpression of ATPIF1 in mice was found to be sufficient to reprogram the metabolism. Such reprogramming has been linked to increased mtROS production and, consequently, to activation of two pathways: nuclear factor-κB (NF-κB) and AMP-activated protein kinase (AMPK). Transgenic mice were healthy but showed an increased resistance to oxidative stress and inflammation, which may be due to the activation of antioxidant defenses as a consequence of higher basal oxidative stress [37, 38], as discussed in the "Cell death" section. Both examples illustrate the complexity to directly link mitochondrial oxidative metabolism with mtROS production.

3.2 Mitochondrial DNA alterations increase ROS production in tumors

Mitochondrial DNA (mtDNA) encodes subunits of ETC Complexes I, III, IV, and V, mitochondrial transfer RNAs, and mitochondrial ribosomal RNAs [1, 2]. Compared to nuclear DNA, mtDNA is more sensitive to mutations owing to the facts that DNA repair mechanisms are limited in mitochondria (although recent evidence suggested the presence of most nuclear DNA repair mechanisms in these organelles [39]) and to the absence of histones that makes mtDNA more accessible to damage [2]. Nevertheless, each cancer cell contains thousands of mtDNA copies, and the frequency of mutated compared to total mtDNA, i.e., the degree of heteroplasmy, considerably impacts the expression of a given mutation [1, 2].

Specific polymorphisms and mutations of mtDNA have been associated with an increased risk of tumor development and progression in humans [40, 41], and frequent germline and somatic mtDNA mutations have been observed in cancer [1, 2, 42, 43]. However, as mtDNA is mother-inherited and because no bias towards maternal inheritance of tumor predisposition has been reported so far in familial cancer syndromes [44], tumor-associated mtDNA mutations are more likely to contribute to tumor promotion than to tumor initiation. In cancer cell lines, several mtDNA point mutations and small (21 bp) deletions (e.g., in genes encoding Complexes I, III, and V subunits) partially impairing the ETC function have been reported to increase mtROS production and to often induce a glycolytic compensation for ATP production, both of which could contribute to tumor progression [1, 2, 44–48]. Conversely, experimentally induced mtDNA depletion is known to decrease cancer cell proliferation *in vitro* and in mice, even when cells are supplemented with nutrients requiring ETC activity for synthesis [1, 49, 50]. Particular cases are oncocytomas that are benign tumors characterized by mtDNA mutations leading to complete homoplasmic inactivation of Complex I but rarely progress to malignancy [51–55]. These observations suggest that retention of a minimal ETC activity is required for tumor progression. Unlike mtDNA point mutations resulting in partial ETC dysfunction and higher mtROS levels, strong impairment of the ETC activity upon large (4.7 kb) mtDNA deletions did not raise mtROS levels in cancer cell lines [44, 45]. In a similar way, tumor-promoting mtROS production might be impaired in mtDNA-depleted cancer cells and in oncocytoma cells,

although this hypothesis requires further investigation. Thus, mtDNA mutations could promote tumor progression through increasing mtROS production, as long as a minimal ETC activity is maintained.

Interestingly, mtDNA alterations have also been linked to chemoresistance. Alkylating agents can cause damage to mtDNA, which can promote cancer cell survival to therapy. This has been exemplified in glioblastoma (GBM), where resistance to temozolomide was shown to alter mtDNA in such a way that it decreased the activity of Complex I and increased the activity of Complexes II, III, and IV [56], resulting in a reduced proton leak and mtROS production while improving ETC coupling and, therefore, OXPHOS-dependent ATP production. Increased Complex IV activity implied a low cytochrome c/cytochrome c oxidase ratio that reduced the cytochrome c pool available to initiate apoptosis. Hence, cytochrome c oxidase was identified as a potential marker of poor prognosis in high grade GBM, patients, as well as a potential target for therapy [57]. Depleting cytochrome c oxidase chemosensitized the cells to temozolomide [58]. Based on these observations, one can propose that mtDNA could contribute to the high adaptability of cancer cells to microenvironmental and pharmacological stresses.

3.3 Microenvironmental influences on ROS production in tumors

Conditions inherent to the tumor microenvironment can influence the activity of mitochondrial enzymes and mitochondrial $O_2^{\bullet-}$ production. For instance, hypoxia stimulates $O_2^{\bullet-}$ release from Complex III and, thereby, initiates hypoxia-inducible factor (HIF) signaling in a prolylhydroxylase (PHD)-dependent way [2, 8, 9, 11, 13, 14, 59–61]. Nutrient deprivation in general and glucose starvation in particular, both of which can arise in tumors from combined poor perfusion and high metabolic demand, further stimulate mtROS production [11, 62, 63]. In the opposite situation, nutrients in excess and/or accumulation of metabolic intermediates also stimulate mtROS production by the ETC [11]. Given the different modes of mtROS production (see "Mitochondrial ROS generation and diffusion" section), nutrient deprivation would slow down, and nutrient excess would increase, the basal activity of the ETC [8, 11]. Alternatively, nutrients in excess could lead to the accumulation of reduced ubiquinone while maintaining a high mitochondrial potential [8, 11]. Of note, among other nutrients, O_2 availability directly impacts ETC activity, and $O_2^{\bullet-}$ generation is thermodynamically and kinetically promoted by high O_2 levels [8]. Finally, alterations of the metabolic activities of other mitochondrial enzymes, such as proline dehydrogenase during nutrient deprivation and α-ketoglutarate dehydrogenase during excess nutrient availability, could further contribute to mtROS production [8, 11].

3.4 ROS amplification loops in tumors

In endothelial cells, mtROS have been shown to stimulate the activity of NAD(P)H oxidases (NOXs), leading to a positive feedback loop of mtROS-induced ROS generation [64, 65]. Whether this mechanism is relevant or not to tumors is unknown, but NOXs are known to significantly contribute to ROS production in cancer cells [26, 66]. Conversely, an extracellular oxidizing environment is sufficient to induce mtROS production by cancer cells through a yet unknown mechanism [67]. This means that ROS release by cancer cells in the surrounding extracellular fluid could induce mtROS in neighboring cells, thereby leading to an amplification loop of ROS-induced mtROS production.

Upregulation and downregulation of mitochondrial K^+ channel expression can both induce ROS production. Indeed, a reduced flux of K^+ hyperpolarizes the IMM, causing an alteration of the oxidative states of complexes I and III that induce $O_2^{\bullet-}$ production [68, 69], whereas an increased K^+ flux would induce IMM depolarization. In order to prevent the osmotic swelling that would be caused by water molecules following K^+ ions, the mitochondrion has to counterbalance the influx of positive charges with an efflux of H^+ through ATP synthase, a process previously known as a futile cycle. The increased activation of the ETC that ensues increases mtROS production [70]. ROS accumulation can further trigger a transient opening of mitochondrial permeability transition pores (mPTP) that enhances mtROS production [70, 71].

3.5 ROS detoxification in tumors

Whatever their origins, ROS levels are determined by the balance between ROS production and inactivation. In this context, antioxidant defense systems are often saturated or decreased in tumors [1, 11, 72], despite, e.g., constitutive activation of Nrf2 [1, 2, 73] and higher PPP activities [32, 74, 75]. An imbalance between ROS production and detoxification can thus also explain why most cancer cells are characterized by higher (mt)ROS levels.

High ROS levels contribute to tumorigenesis [2]. Despite this, cancer cells need a minimum level of ROS detoxification to avoid cell death, as recently reviewed in [76]. The proliferation and progression of acute myeloid leukemia (AML) is promoted by high level of mtROS [77]. The mechanism by which AML cells increase mtROS production involves protein kinase C ε (PKCε): PKCε silencing or inhibition was proven to negatively affect both AML onset and cell proliferation in genetically engineered mice due to an increase in ROS

(including mtROS) production. The protumoral phenotype was recapitulated by inhibiting mitochondria-resident SOD2 [78], further highlighting the key role of mtROS in the initiation and progress of AML [77].

4 Consequences of elevated mtROS levels in tumors

4.1 Genetic effects

Owing to its proximity to mtROS production sites, the absence of protective histones, and limited mtDNA repair mechanisms, mtDNA is a primary mtROS target. Because mtDNA encodes mitochondrial ETC complex subunits and because mutations in such genes can lead to increased electron leakage, mtROS are thought to increase their own production in a positive feedback loop [2, 11]. When a limited amount of ROS reaches the cell nucleus, ROS can induce nuclear DNA mutations that could contribute to genomic instability, tumor initiation, and tumor progression (Fig. 2) [2, 79–81]. Exogenous ROS and NOX-generated ROS further stimulate DNA methyltransferase activities, leading to hypermethylation/silencing of tumor suppressor genes [66, 82]. But whether mtROS influence tumor progression through similar epigenetic mechanisms still requires investigation.

4.2 Cell death

High mtROS levels or a prolonged exposure to mtROS leading to excessive/cumulative damage (DNA mutations, lipid peroxidation, and protein oxidation) can ultimately induce cell death [2]. High mtROS levels can also trigger apoptosis independently of DNA damage. ROS can indeed react with the MPTP and cytochrome *c* [2, 83, 84]. Oxidized MPTP becomes permeant to Ca^{2+} and H^{+}, which are released from the IMS to the cytosol, and oxidized cytochrome *c* detaches from the MIM and can therefore be transferred to the cytosol after permeabilization of the mitochondrial outer membrane. Both events initiate the apoptotic cascade [2, 83, 84].

4.3 Prosurvival effects

Surprisingly, moderately increased mtROS levels have been reported to prolong the lifespan of yeasts and *Caenorhabditis elegans* nematodes, which has been attributed to an

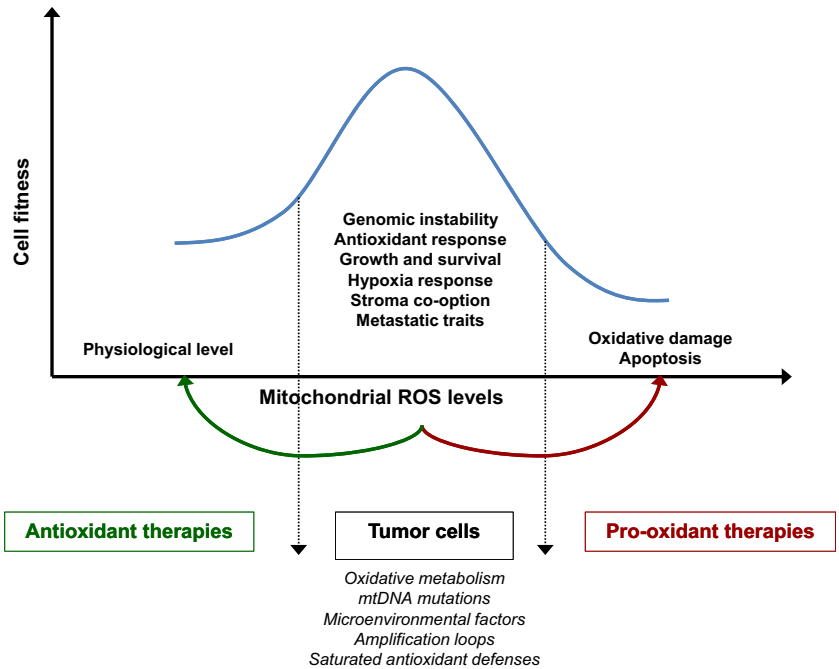

Fig. 2 Increased mitochondrial ROS in cancer: a double-edged sword. Cancer cells often have higher mitochondrial ROS levels than healthy cells, which can be due to stimulation of oxidative metabolism by oncogenic pathways, mitochondrial DNA (mtDNA) mutations, microenvironmental factors such as oxygen or nutrient deprivation, positive feedback loops promoting ROS production, and saturated antioxidant defenses [2, 9, 11]. In a certain window of concentration, mtROS promote tumor progression by inducing DNA mutations and genomic instability, upregulating antioxidant defenses (Nrf2 signaling), stimulating proliferation and survival pathways (Akt, nuclear factor-κB, extracellular-regulated kinases 1–2, autophagy), contributing to the adaptation of the cells to hypoxic conditions (hypoxia-inducible factors [HIF] signaling), co-opting the stroma (HIF-1 signaling), and promoting cancer cell dissemination (Snail, nuclear factor κB, and HIF-1 signaling) [2, 9, 11, 44]. If present at a too high concentration, mtROS are detrimental and damage nucleic acids, lipids, and proteins and induce apoptosis. Thus, two opposite approaches aiming at reducing or increasing ROS levels in order to impair ROS signaling or to sensitize cancer cells to oxidative stress, respectively, have been considered in cancer therapy [2]

upregulation of antioxidant defense systems analogous to mammalian Nrf2 and Prx2 and, thus, to a higher resistance to environmental oxidative stress [85–87]. Similarly, in tumors, sublethal levels of mtROS can activate signaling pathways promoting cancer cell survival, proliferation, and tumor growth, including (*i*) Akt, through inhibition of redox-sensitive phosphatase and tensin homolog (PTEN) [2, 9]; (*ii*) NF-κB, through activation of redox-sensitive tyrosine kinase Src under hypoxia [72]; (*iii*) the extracellular signal-regulated kinase (ERK) 1–2 pathway in anchorage-independent conditions [28, 74]; and (*iv*) Atg4, an enzyme promoting autophagy, e.g., for the elimination of damaged mitochondria [9, 88, 89].

4.4 HIF activation

In tumors, a major effect of mtROS is related to the stabilization of HIF-α subunits under hypoxic conditions. While HIF PHD inactivation by limited oxygen availability is well accepted to stabilize HIF-1α and HIF-2α proteins, hypoxia-induced mtROS generation has been proposed as a complementary mechanism [13, 14, 59–61, 90, 91]. mtROS produced at ETC Complex III indeed oxidize Fe^{2+} to Fe^{3+} at the PHD catalytic site, which inactivates the enzyme. Similarly, exogenous ROS are sufficient to stabilize HIF-α subunits in a PHD-dependent manner, leading to a ROS-induced pseudo-hypoxic response [14]. Factor inhibiting HIF (FIH), a Fe^{2+}-dependent repressor of HIF transcriptional activity, is even more sensitive than PHD to ROS-induced inhibition, further contributing to mtROS-induced HIF signaling [92]. In addition, pseudo-hypoxic HIF activation in SDH- and fumarate hydratase (FH)-mutated cancer cells is likely to have a redox component [2]. The SDH complex belongs both to the TCA cycle and to the ETC, so that SDH mutations can induce electron leakage and $O_2^{\bullet-}$ release at ETC Complex II [2]. FH-mutated cells accumulate fumarate, an electrophilic metabolite that can form adducts with GSH, thereby depleting GSH stocks [2]. Both mechanisms could contribute to ROS-induced HIF signaling.

The HIF transcriptional response allows cancer cell adaptation to hypoxic conditions with an upregulation not only of genes encoding glycolytic enzymes, pH regulators, and pro-angiogenic factors but also of genes encoding proteins involved in the antioxidant response. One of these proteins is cytochrome *c* oxidase (COX) 4–2, an alternative subunit of ETC Complex IV that optimizes O_2 consumption for ATP production and decreases mtROS production upon limited O_2 supply [93]. Another protein induced by HIF is pyruvate dehydrogenase kinase 1 (PDK1) that inhibits pyruvate dehydrogenase (PDH), thus decreasing oxidative pyruvate metabolism in mitochondria [94]. In addition, oscillation of HIF-1-induced pyruvate kinase M2 (PKM2) [95] between an active tetrameric form and an inactive dimeric form further fine-tunes the distribution of glucose-6-phosphate between glycolysis and the PPP. This control is allosteric: PKM2 is activated when glycolytic intermediate fructose-1,6-bisphosphate accumulates upstream of PKM2 and inhibited when pyruvate-derived alanine accumulates downstream of PKM2 [96, 97]. Furthermore, oxidation of a redox-sensitive cysteine residue stabilizes the inactive/dimeric form of PKM2 and increases the PPP flux, thus stimulating, e.g., NADPH production for antioxidant defenses [75]. HIF-1 also induces the expression of Bcl-2-interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L) protein that stimulate mitochondrial autophagy [98, 99], thus reducing the amount of ROS-producing organelles. HIF-2 further induces *GPx1*, *SOD1* and *SOD2* transcription [100].

HIF-1α activation is involved in the pathogenesis of stomach cancer in the presence of *Helicobacter pylori* through a ROS-HIF-1α-aquaporin 3 (AQPR3)-ROS loop [101]. Conversely, inhibition of HIF-1α synthesis is responsible for the cytotoxicity of vosaroxin (a topoisomerase 2 inhibitor) through the AMPK-sirtuin 3 (Sirt3)-HIF-1α pathway [102]. In short, vosaroxin activates AMPK that, in turn, increases the expression of Sirt-3 through the activation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α). Higher levels of Sirt-3 were correlated to a reduction of HIF-1α protein levels. Reduced HIF-1α and consequent ROS production caused cytotoxic effects that were reduced upon Sirt-3 silencing.

Altogether, mtROS-induced activation of the HIF transcriptional program participates in cancer cell survival and in the maintenance of redox homeostasis under hypoxia. Conversely, anticancer effects of ROS-inducing therapies might be limited by ROS-dependent HIF-1α activation [103–105]. In that case, targeting HIF signaling could potentially have synergistic effects with these treatments. To date, anti-HIF therapy is in progress, but no candidates successfully passed Phase 2 clinical trials yet [106].

4.5 Effects on stromal cells

ROS also induce changes in stromal cells. First, oxidative cancer cells that use lactate as a fuel can induce a glycolytic phenotype in cancer-associated fibroblasts (CAFs) through soluble factors in a ROS- and HIF-1-dependent manner [107, 108]. This form of metabolic co-option promotes lactate production by CAFs to fuel oxidative cancer cell metabolism. Similarly, cancer cells can activate NF-κB and AP-1 in immune cells in a ROS-dependent fashion to release pro-inflammatory cytokines supporting cancer cell survival [11]. In both cases, whether ROS shuttle from cancer cells to stromal cells and whether they originate from mitochondria has not yet been firmly established. However, mitochondria are unlikely the source of ROS because of the high chemical reactivity of ROS and the multiple obstacles (e.g., membranes) and targets (e.g., thiols, lipids) along the way. In general, ROS-mediated tumor-stroma crosstalk serves stroma co-option by cancer cells.

4.6 Effects on autophagy

Autophagy is considered to be protective against cancer initiation by reducing DNA damage due to ROS. This effect is obtained, at least in part, by recycling defective mitochondria, which are the main ROS-producing organelles. However, after tumor initiation, autophagy promotes cancer survival notably by controlling ROS overproduction [109] and, probably, by mitigating mitochondrial dysfunctionality caused by mtROS-induced mtDNA damage. As previously mentioned, autophagy can be induced by mtROS through the mtROS/HIF-1/BNIP3 and BNIP3L pathway [98, 99]. However, the nearest and most likely affected target of mtROS is cysteine protease Atg4, the ROS-induced inhibition of which promotes mitophagy [110], i.e., a mitochondria-specific autophagic process. ROS-dependent NF-κB activation induces the expression of p62, another protein-promoting mitophagy [111].

4.7 Effects on metastasis

That mtROS can promote tumor metastasis was first evidenced in experiments using mitochondria null cancer cells: murine melanoma and breast cancer cells lacking mtDNA were observed to acquire mtDNA from host cells during metastasis, likely by intercellular mitochondrial transfer [112]. In these models, the progressive recovery of mtDNA allowed cancer cells to efficiently metastasize in mice, which was accompanied by a concomitant increase in (mt)ROS production. Along the same line, mtDNA mutations leading to higher mtROS production by ETC Complex I were found to correlate with breast and lung cancer cell migration, invasion, and metastasis [1, 44, 113]. Collectively, these observations are suggestive of a prometastatic role of mtROS. Five potential effects have been described to date. First, mtROS promote cancer cell survival and proliferation in anchorage-independent conditions by activating the ERK1–2 pathway [28, 74]. Second, in several models, mtROS were shown to induce an epithelial-mesenchymal transition transcriptional program, notably by activating Snail, HIF-1, and Met [29, 113, 114]. Third, mtROS can activate NF-κB, which stimulates the release of matrix metalloproteinase 2, angiogenesis, and cancer cell invasion [48]. Fourth, mtROS promote prostate cancer cell engraftment in bones because it increases focal adhesion kinase (FAK) phosphorylation and enhances the release of acidic fibroblast growth factor (aFGF) [115]. Fifth, mtROS were found to promote experimental and spontaneous metastasis in a murine model of lung carcinoma [44, 45]. In this model, mtROS upregulated the expression of anti-apoptotic protein myeloid leukemia cell differentiation-1 (MCL-1) and induced HIF-1 signaling and vascular endothelial growth factor expression in the absence of a glycolytic switch. The metastatic phenotype was reversed by the administration of *N*-acetyl-*L*-cysteine (NAC) or the GPx mimetic, ebselen. In parallel, mtROS generation promoted by complex I or III dysfunction (induced by low-dose rotenone and a shRNA targeting UqcrB, respectively) has been shown to promote experimental metastasis, a response that was repressed by mitochondria-targeted antioxidants mitoQ and mitoTEMPO [116]. These findings were recapitulated in a spontaneous breast cancer model treated with mitoTEMPO.

It is also tempting to link mtROS production with pH regulation, as (*i*) increased mtROS levels following glucose deprivation posttranslationally induce the expression of monocarboxylate transporter 1, and, thereby, cancer cell migration towards glucose, possibly *via* a pH-dependent mechanism [62], and, reciprocally, (*ii*) acidic priming of cancer cells can transiently stimulate their migration, invasion, and lung colonization in a ROS-dependent manner [117]. Except from the latter study, there is a lack of data linking mtROS to pH modulation in the context of tumor metastasis.

Conversely, there is an equivalent number of studies reporting that (mt)ROS do not promote, but rather impair, tumor metastasis. Indeed, cancer cell de-adhesion from the extracellular matrix induces ROS production, which can, ultimately, trigger anoikis and, thus, limit the metastatic success rate [118, 119]. To face high ROS levels upon inadequate anchorage, cancer cells upregulate the expression of SOD2 [120], increase NADPH production by the PPP [32, 121] and by the folate pathway [119], and consume GSH stocks [119]. Accordingly, NAC and vitamin E analogue Trolox stimulated cancer cell migration, invasion, and metastasis in mouse melanoma models [119, 122]. These responses were independent of cancer cell proliferation and primary tumor growth. They were attributed to oxidative stress management [119, 122] and to a stimulation of RhoA small GTPase signaling by these general antioxidants [122]. The corollary is that increasing oxidative stress would impair metastasis. Indeed, silencing NADPH-producing enzymes of the folate pathway and inhibiting dihydrofolate reductase with low doses of methotrexate decreased the number of circulating cancer cells and the occurrence of metastases without having an effect on primary tumor growth in murine models of melanoma [119]. Thus, the role of (mt)ROS in metastases appears to be dual and, most probably, tumor and oncogenic context-specific.

5 Redox-based therapies against cancer

The duality of ROS pro- and anticancer effects explains why both antioxidant and pro-oxidant strategies have been considered for cancer treatment, for prevention, or for treatment, alone or in combination (Fig. 2). Signaling or damaging effects of ROS depend on their nature, their subcellular

localization, their level, the duration of their production, and cell sensitivity.

The use of general antioxidants, including NAC, β-carotene, vitamin C, vitamin E, and analogues, led to contradictory effects in murine models of tumorigenesis and tumor growth [123, 124]. No or even deleterious effects were observed when general antioxidants were tested as prophylactic agents [125, 126] or as adjuvant treatments with conventional therapies in humans [127–130]. Because of adverse effects, cancer patients are currently recommended to avoid antioxidant supplementation in the absence of a nutritional deficiency [26, 126, 130]. Identification of responsive patients [126, 131], adequate dose, frequency and time frame of administration, characterization of interferences with conventional therapies [130], and development of antioxidants targeted to specific subcellular compartments and targeting specific ROS [2, 132, 133] represent potential improvements for antioxidant cancer therapies. In this context, mitochondria-targeted antioxidants (mtAOs) mitoTEMPO and mitoQ are of particular interest. They were developed by coupling a stable pyridine *N*-oxide radical or ubiquinone, respectively, to a lipophilic and positively charged triphenylphosphonium (TPP) moiety, thereby allowing their accumulation in the negatively charged mitochondrial matrix [132]. In cancer, mitoTEMPO was shown to reduce mtROS-induced Akt and HIF-1 signaling and to decrease melanoma xenograft growth in mice at high doses [134]. Both agents repressed cell migration, invasion, and metastasis in several tumor types in mice [116]. In another study, mitoTEMPO reduced the migration of breast cancer cells overexpressing mitochondrial anti-apoptotic protein G1P3 (IFI6), but NAC and polyethylene glycol (PEG)-catalase produced similar effects, suggesting a concerted effect of mtROS and ROS on cell migration in this model [135]. In a mouse model of chemical hepatocarcinogenesis, non-mitochondria-targeted antioxidants, NAC and Trolox, were effective in preventing tumorigenesis, while mitochondria-targeted antioxidants SS-31 and mitoQ promoted tumorigenesis [136]. NAC and Trolox indeed activated the ATM/ATR DNA repair pathways, which SS-31 and mitoQ inactivated. SkQ, another mitochondria-targeted antioxidant structurally similar to mitoQ, reduced the proliferation of rhabdomyosarcoma and fibrosarcoma cell lines [137]. However, in this system, NAC and Trolox produced similar effects.

An alternative anticancer approach consists in selectively inducing oxidative stress in cancer cells by taking advantage of their generally higher basal ROS levels and relatively saturated antioxidant defense systems. While part of the cytotoxic effects of radiotherapy and of some conventional chemotherapeutic agents is actually mediated by this mechanism, other molecules are under evaluation for this specific purpose, such as the menadione-ascorbate combination and β-phenylethylisothiocyanate [2, 130, 134, 138–142]. Dichloroacetate (DCA), a PDK1 inhibitor, has also been evaluated as a mitochondria-targeted pro-oxidant [143]. However, until now, DCA yielded poor clinical responses, indicating that inducing a lethal production of mtROS may be challenging. It is also important to note that an antioxidant pretreatment strongly reduced the effectiveness of cytotoxic ROS-inducing treatments in different (H-, K-, B-) rat sarcoma virus (RAS)-mutated cell lines [144, 145]. This, again, highlights that any clinical application of antioxidants should be carefully evaluated to avoid antagonistic effects on the activity of conventional treatments.

Photothermal therapy and photodynamic therapy are two new approaches in cancer therapy that are finding new applications through mitochondrial targeting. Nanomaterials (e.g., gold, platinum, carbon, iron oxide, titanium oxide nanoparticles) and organic molecules (e.g., cyanidines) can be chemically linked to mitochondria-targeting moieties, such as TPP, in order to selectively accumulate inside mitochondria. These systems are reactive to photo-irradiation or near infrared (NIR) irradiation and, after irradiation, directly or indirectly generate mtROS to induce apoptosis [146] or necroptosis, which can involve mitophagy [147–149]. Some examples are mito-CCy [150], mito-CIO [151], protein-ruthenium hybrids [152], and indocyanine green (an FDA-approved photosensitizer) [153]. This approach allows targeting of cancer cells with a good selectivity. It is expected to be less mutagenic for healthy cells than γ-irradiation, as nuclear DNA should not be directly damaged by lower energy radiations. However, DNA may be indirectly damaged through ROS generation, indicating that short- and long-term side effects of this therapy need to be further studied. In addition, limited tissue penetration (i.e., deeper than UV and visible light but more superficial than γ rays) of the NIR irradiation may result in an inefficient stimulation of photosensitive systems in case of deep or blood-perfused tumors [154].

6 Conclusions

Redox-based therapies, alone or in association with chemo- and radiotherapy, may lead to a new strategy for cancer treatment and prevention. However, many factors like microenvironment, antioxidant-specific mechanisms of action, the stage of the tumor, its location, and ROS and mtROS levels in the tumor affect the efficacy of the therapy. Furthermore, studies are needed to establish the conditions in which each class or specific antioxidant is effective to avoid inducing tumorigenesis or promoting tumor growth instead of preventing/treating it. Therefore future clinical application of redox-based antioxidants as prophylaxis or as adjuvant treatments should be oriented towards a personalized therapy.

Among all the studies considered in this review, the biological mechanisms underlying observed effects are rarely fully explained. Evidence highlights links between mtROS

and several pathways, including those involved in cell cycle regulation, DNA repair, cytoskeleton reorganization, RAS, and HIF-1 signaling [134, 136, 137, 144, 145]. Often, non-targeted and mitochondria-targeted antioxidants produce similar effects, suggesting that mtROS are key contributors to tumor progression. Some activities are dependent on the cell line or tumor studied. Future experiments should thus take into account these variables and include in the experimental conditions non-mitochondrial-targeted antioxidants in order to address effects only related to mtROS reduction. A comparison of models that respond (or not) to non-mitochondria-targeted antioxidants *versus* models that respond only to mitochondria-targeted antioxidants is necessary in order to identify the mediators responsible for (mt)ROS-dependent activation of several signaling pathways. A deeper understanding of the biological mechanisms of action of mtROS is the necessary key to explain contradictory results and ease the way of redox-based therapies towards clinical applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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