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Review

Mitochondrial reactive oxygen species cause major oxidative mitochondrial DNA damages and repair pathways

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Mitochondria-derived reactive oxygen species (mROS) are produced at a variety of sites and affect the function of bio-molecules. The anti-oxidant system from both mitochondria and cytosol tightly coordinate to maintain the redox balance of cells and reduce damage from mROS. Mitochondrial DNA (mtDNA) are highly susceptible to mROS, and are easily oxidized to accumulate DNA modifications. Frequent oxidative damages in mtDNA have been associated with neurological degeneration, inflammasomes, tumorigenesis, and malignant progression. Among mitochondrial DNA repair pathways, the base excision repair pathway has been extensively characterized to remove some of oxidative damages in mtDNA as efficiently as the nuclear base excision repair. The implications of other pathways remain unclear. This review focuses on: (i) Sources of mROS and the antioxidant system to balance redox status; (ii) major mtDNA lesions or damages from mROS-mediated oxidation and the reported repair pathways or repairing factors; (iii) cellular response of oxidized mtDNA and methods to identify oxidatively generated DNA modifications in pathological conditions. DNA damages caused by mROS have been increasingly implicated in diseases and aging, and thus we critically discuss methods of the oxidative modifications evaluation and the complexity of non-canonical DNA repair pathways in mitochondria.

Keywords. DNA damage; mitochondria; oxidative modifications; reactive oxygen species; redox balance; repair

Abbreviations: mROS, mitochondrial ROS; TCA, tricarboxylic acid; ROS, reactive oxygen species; ETC, electron transport chain; mtDNA, mitochondrial DNA; Prxs, peroxiredoxins; BER, base excision repair; OGG1, 8-oxoGua by DNA glycosylase

1. Introduction

Reactive oxygen species (ROS) are sourced from cellular compartments including phagosomes, peroxisomes, endoplasmic reticulum, plasma membranes, and mitochondria to mediate cellular signaling for cell division, death, etc. Mitochondria, the location of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS), supplies most of the ATP and generates substantial amounts of mitochondrial ROS (mROS) (Shadel and Horvath 2015). Antioxidant systems in mitochondrial compartments scavenge ROS to mitigate oxidative damages. The balance between ROS and the capacity of antioxidants determine the redox status in mitochondria as well as redox signaling of the whole cell under both healthy and pathological conditions.

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Primary reactive species including superoxide anion (O_2•−), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) can be generated by mitochondria. These mitochondrial ROS (mROS) are the metabolic products of energy production (Shadel and Horvath 2015; Murphy 2009; Mailloux 2015; Diebold and Chandel 2016; Gammella *et al.* 2016). Under the condition of single electron leakage, electron transport chain (ETC) that transfers electrons from electron donors to electron acceptors via redox reactions, coupling with protons (H+ ions) transport across a membrane, will be the source of O_2•− and it is released in both the matrix and inter-membrane space. Intermembrane-space O_2•− that reaches the cytosol has been reported to modify genomic DNA (Lemarie *et al.* 2011; Quinlan *et al.* 2013; Nickel *et al.* 2014; Han *et al.* 2003). Mitochondria-localized NADPH-oxidase 4 and monoaminoxidase (MAO) are generators of ROS, in the form of O_2•− and H_2O_2, respectively. Enzymatic involvement via flavin-containing prosthetic groups in fatty acid oxidation also contributes to ROS generation. The Fenton reaction can generate •OH with transition metals (Sabharwal and Schumacker 2014; Vallyathan *et al.* 1998; Donaldson *et al.* 2003). The accumulation of ROS is deleterious and undermines the normal physiology of cells. To prevent the detrimental effects brought by ROS, antioxidant systems function synergistically to sustain the redox balance (Nickel *et al.* 2014; Zorov *et al.* 2014; Chio and Tuveson 2017).

Mitochondrial DNA (mtDNA) of most organisms is a circular molecule. The human mtDNA consists of genes encoding 13 polypeptides, 22 tRNAs and 2 rRNAs, with only 600 non-coding nucleotides (Anderson *et al.* 1981). With such high density of genes, mtDNA lesions often cause aborted transcripts either via site-blocking or coding nucleotide modifications. Damages on genes from the oxidative stress interfere with its related physiological processes. DNA mutations are introduced predominately at guanine residues of mtDNA. Some mutations in mtDNA in turn induce escalating ROS generation, and even drive tumorigenesis or promote tumor progression (Woo *et al.* 2012; Brandon *et al.* 2006; Alexeyev *et al.* 2013). In addition to that, mitochondrial oxidants cause double-strand breaks and chromosomal translocations which are related to decreased cell viability or tumor-inducing behaviors (Samper *et al.* 2003; Radisky *et al.* 2005).

Oxidative stress generates most species of chemical modifications in DNA. Accumulated mitochondrial oxidants, susceptibility of mtDNA to ROS attacks, and lack of nucleotide excision repair (NER) result in the complex chemical modifications in the mitochondria. However, far fewer oxidative modification species have been identified in mtDNA in contrast to nuclear DNA, due to current analysis methods. In this review, we summarize ROS generation in the mitochondria, the characterized mtDNA damages and repairing pathways, and cellular responses triggered by oxidized mtDNA, and we discuss the unknown repair mechanism constituted by some proteins. These are critical for us to portrait the DNA repair network in the mitochondria and discover the unknown modification types. Furthermore, the course to advance the techniques exploited for base modification identifications has been addressed here, which is important for establishing the link between modifications and diseases.

2. Oxidative stress from mitochondria and antioxidant defense

2.1 *ROS generation in mitochondria*

In mitochondria, while electrons from NADH and $FADH_2$ produced in TCA oxidizing reactions are transferred to ETC, and ultimately to molecular O_2, the free energy released is converted and stored in ATP. The membrane potential, the driving force for ATP synthesis at complex V, results from pumping of proton at Complexes I, III and IV, coupled with step-wise electron transfer. Among ETC complexes, Complexes I and III are composed of flavin prosthetic groups, Fe/S clusters. and generate O_2•− by transfer an electron to 2–5% dioxygen O_2 in the following manners (Wippich *et al.* 2001): (1) Electron leakage at Complexes I and III under normal function of the ETC, e.g. under extracellular acidosis, increased OXPHOS activities cause leakage increase and therefore rising mtROS production (Murphy 2009); (2) under the circumstance of low respiration rate or high NADH/NAD+ ratio, delay during the sequential transfer induces O_2•− production at the NADH oxidation site of Complex I (Murphy 2009; Quinlan *et al.* 2013); (3) reverse electron transfer from ubiquinone to Complex I. With the steep electric potential across mitochondrial inner membrane and abundant ubiquinone, the route of electrons in the inner membrane are impeded, which therefore increases chances of O_2 contact in Complex I (Murphy 2009; Sabharwal and Schumacker 2014); (4) hypoxia induces increase in O_2•− production at Complex III. With the unknown mechanism, under low O_2 availability, electrons accumulated at sites Q0 and Qi of Complex III generate O_2•− (Murphy 2009).

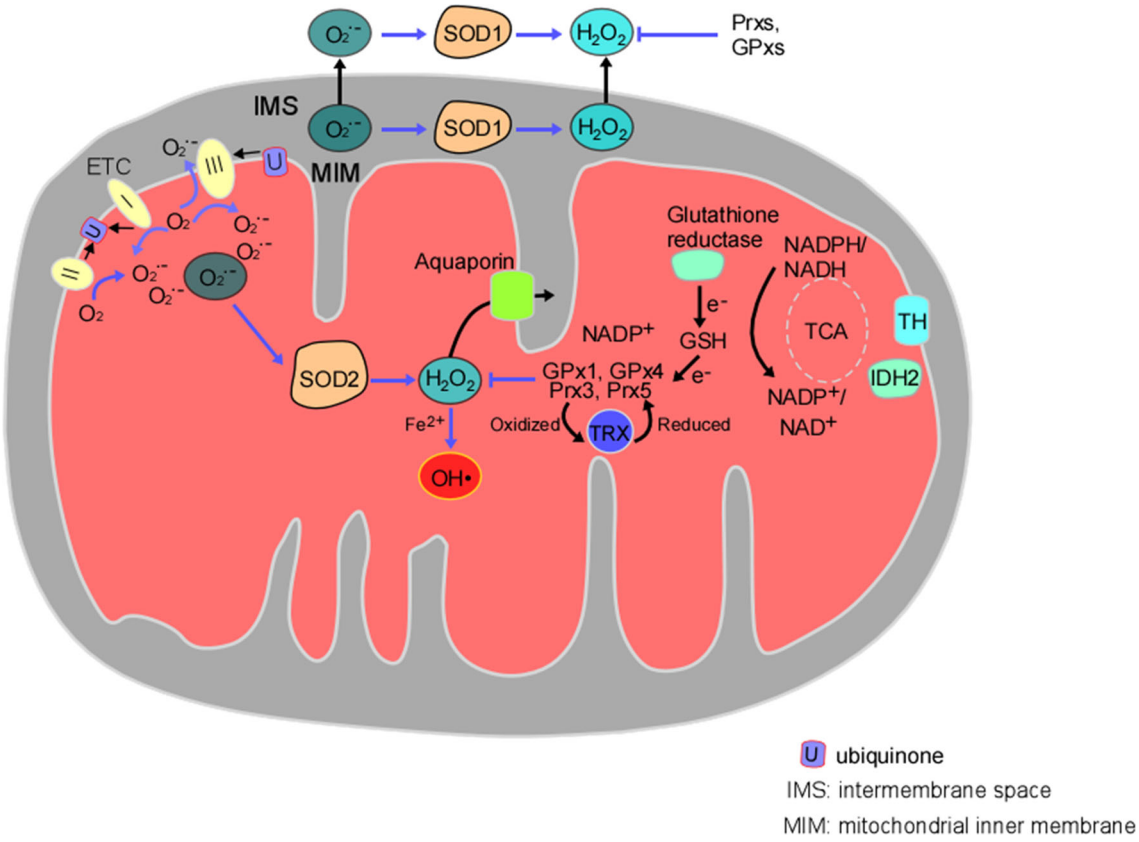


Figure 1. mROS generation sites and catalytic antioxidant system. ROS generation occurs via single electron leakage in ETC under specific conditions. Superoxide O_2•− generated from complexes I, II and III, either releases in the matrix compartment or to the intermembrane space by complex III. Enzymes in TCA can potentially generate ROS. Superoxide O_2•− is converted to H_2O_2 by superoxide dismutase in the matrix (SOD2; also known as MnSOD), or SOD1 in the intermembrane space. The H_2O_2 is degraded by peroxidases: glutathione peroxidases (GPxs), with the reducing equivalents from the oxidation of reduced glutathione (GSH). Glutathione reductase reduces oxidized glutathione (GSSG), gaining its equivalents from NADPH oxidation. Peroxiredoxins (Prxs), reduced primarily by thioredoxins (TRX). H_2O_2 can potentially leak to the intermembrane space and the cytosol during excessive ROS generation or under the discordant antioxidant condition. H_2O_2 without being neutralized can be readily converted into •OH in the presence of Fe^{2+} and immediately reacts at its production site. TH, mitochondrial trans-hydrogenase; IDH2, iso-citrate dehydrogenase 2.

Among all these ROS generating sites, effects on redox status of subcellular compartments are different. While superoxide from inner mitochondrial membrane is readily released into the intermembrane space (IMS) and further into cytosol via the voltage-dependent mitochondrial anion channel due to electric field formed within the membrane (figure 1), O_2•− derived from prosthetic groups towards the matrix, on the other hand, is discharged into the matrix (Alexeyev *et al.* 2013; Quinlan *et al.* 2013).

In the mitochondria, most of the O_2•− are dismutated to H_2O_2 by manganese superoxide dismutase (MnSOD) in the mitochondrial matrix; peroxynitrite is also yielded when O_2•− reacts with nitric oxide (Murphy 2009). H_2O_2 is more stable than O_2•− and a highly diffusible second messenger, also capable of crossing the mitochondrial membrane through aquaporin family proteins (Bienert *et al.* 2007). These ROS accumulate in the cytosol and have been shown to modify DNA and are involved in cell differentiation, oxygen cell sensing, and adaptive immunity (Sena and Chandel 2012; Muller *et al.* 2004). Mitochondrial ROS has a damaging role and cellular signaling properties. H_2O_2 crosses the membrane through aquaporins as superoxide through anion channels (figure 1) (Han *et al.* 2003; Bienert *et al.* 2007). In the mitochondria, spontaneously generated or SOD-dependent H_2O_2 can be converted to the •OH via the Haber-Weiss Fenton reaction (Kehrer 2000; Cadet and Wagner 2013). During the reaction, Fe^{2+} plays as a reducing agent. •OH is extremely unstable and culminates in the oxidative reactivity with cell components, proteins, lipids, and nucleic acids (Sabharwal and Schumacker 2014; Cohn *et al.* 2006).

TCA is a series of enzymatic reactions to release stored energy through the oxidation of acetyl-CoA from carbohydrates, fats, and proteins, into adenosine triphosphate (ATP) and carbon dioxide. During the processes, NAD^+ and flavin adenine dinucleotide carry high-energy electrons from intermediary metabolites to the electron transport systems in the inner mitochondrial membrane. Enzymes with flavin prosthetic groups for electron transfer may also be the ROS generating sites, for example, proline dehydrogenase, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched chain α-ketoacid dehydrogenase, succinate dehydrogenases, and acyl-CoA dehydrogenase (Murphy 2009). Also, 2-oxoadipate dehydrogenase in the mitochondrial matrix, and dihydroorotate dehydrogenase and glycerol-3-phosphate dehydrogenase in the IMS, can be the sites for ROS production in the mitochondria (Murphy 2009).

2.2 *Antioxidant system to balance redox status*

There are enzyme and non-enzyme-based processes coordinating the conversion of highly reactive ROS into less reactive ones. Antioxidant enzymes in the cytosol, IMS, and matrix can neutralize ROS. These independent controls over the redox homeostasis in these sub-compartments (Waypa *et al.* 2010; Sabharwal *et al.* 2013), ultimately limit signaling effects and oxidative damage caused by ROS. SOD1, present in cytosol and IMS, is dismutating $O_2\bullet-$ derived from the electron transfer chain, and other locations in the cytoplasm. Mitochondrial SOD2 is responsible for the catalysis of $O_2\bullet-$ from ETC into H_2O_2 mostly in the mitochondrial matrix (Tsang *et al.* 2014; Mohammedi *et al.* 2015).

The H_2O_2 produced by SODs are mostly reduced to H_2O by peroxidases: catalase, glutathione peroxidases (GPxs), and peroxiredoxins (Prxs). In the mitochondrial matrix, catalase, GPx1, GPx4, Prx3, and Prx5 are scavengers of hydroperoxides by supplying electrons (Murphy 2009; Marí *et al.* 2009). GPx1 and GPx4, along with reduced glutathione (GSH), provide electrons, reducing H_2O_2 to water. With electrons removed, GSH is oxidized to a dithiol molecule (GSSG). GSSG distributed both in cytosol and mitochondria will be reduced. With glutathione reductase, the reduction electrons are derived from NADPH (Gordillo *et al.* 2016) (figure 1). Peroxiredoxins are specialized in H_2O_2-scavenging by oxidization of reactive cysteines (Sabharwal *et al.* 2013). The oxidized cysteines are reduced primarily by thioredoxins (TRX), while oxidized TRXs are recycled back to by thioredoxin reductases, which also use NADPH as the source of electrons (Cassidy *et al.* 2015).

Serving as the supplier of reducing equivalents, NADPH is vital for persisting the balanced redox state. In cancer, overconsumption of NADPH results in the chronic oxidative stress on cells. Pentose pathway is an important supplier of NADPH in cytosol, obtaining electrons from glucose-6-phosphate mediated by glucose-6-phosphate dehydrogenase. It reroutes glycolytic pathway to the purines and NADPH production in the cytosol (Blacker and Duchen 2016; Fan *et al.* 2014; Tanaka *et al.* 2015). Another major NADPH source is the process involving conversion of methylene tetra-hydro-folate oxidation to 10-formyltetrahydrofolate. In mitochondria, NADPH is generated by methylene tetrahydrofolate oxidation following serine conversion. Mitochondrial NADPH are also produced by isocitrate dehydrogenase 2 from the TCA cycle and the transhydrogenase system (Fan *et al.* 2014; Smolkova and Ježek 2012). When hydrogen peroxide overwhelms the clearance capacity of anti-oxidant systems, it often causes the oxidation of protein thiol and as a result the cellular signaling will be disrupted (Ida *et al.* 2014).

3. Oxidative damages on mitochondrial DNA

Human mtDNA encodes subunits of the oxidative phosphorylation system, 12S and 16S ribosomal RNAs, and 22 transfer RNAs. Among the 13 polypeptides submits, 7 submits are from Complex I, 1 from Complex III subunit, 3 from Complex IV subunits and 2 from Complex V subunits (Shadel and Horvath 2015). There are dozens of mtDNA copies in each mitochondrion; given each cell contains also multi-mitochondria, the copy number of mtDNA could be around hundreds. The mtDNA, without the protection of nuclear-style chromatin structure, is more susceptible to the attack of ROS generated in the matrix and ETC. In addition, lacking the array of DNA repair pathways also leaves mtDNA with higher risk of damage in comparison with the nucleus with both base excision repair (BER) and NER (Tumurkhuu *et al.* 2016). As a result, the rate of oxidatively generated DNA modifications (ODMs) is much greater than nuclear DNA (Larsen *et al.* 2005). With a hundred different modifications identified *in vitro*, the follow section is to focus on the most frequent ODMs implicated in cellular homeostasis.

3.1 *8-oxo-7,8-dihydroguanine in mitochondria*

Many oxidative-stress-driven nucleobase lesions are mutagenic. The oxidative ROS-induced DNA damages include apurinic/apyrimidinic (basic) DNA sites, oxidized purines and pyrimidines (figure 2), and single-strand and double-strand DNA breaks. One of the most common mtDNA base modifications by oxidative radicals is 8-oxo-7,8-dihydroguanine (8-oxoGua). During the reaction, the hydroxyl radical attacks the guanine ring at its C8 site, forming an 8-hydroxy-7,8-dihydroguanyl radical. The redox status of the environment determines the oxidization or reduction of the radical, either oxidized to 8-oxoGua (Altieri *et al.* 2008; Muftuoglu *et al.* 2014; Roszkowski *et al.* 2011) or reduced to 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) (figure 2A).

In mitochondria, BER handles small, non-distorting lesions 8-oxoGua. All enzymes required for BER are present in mitochondria, and function in a similar manner as in nuclei (Mandavilli *et al.* 2002). In the pathway of a single nucleotide replacement, single-nucleotide base excision repair (SN-BER) is initiated by the attack of 8-oxoGua,8-oxo-7,8-dihydroguanine

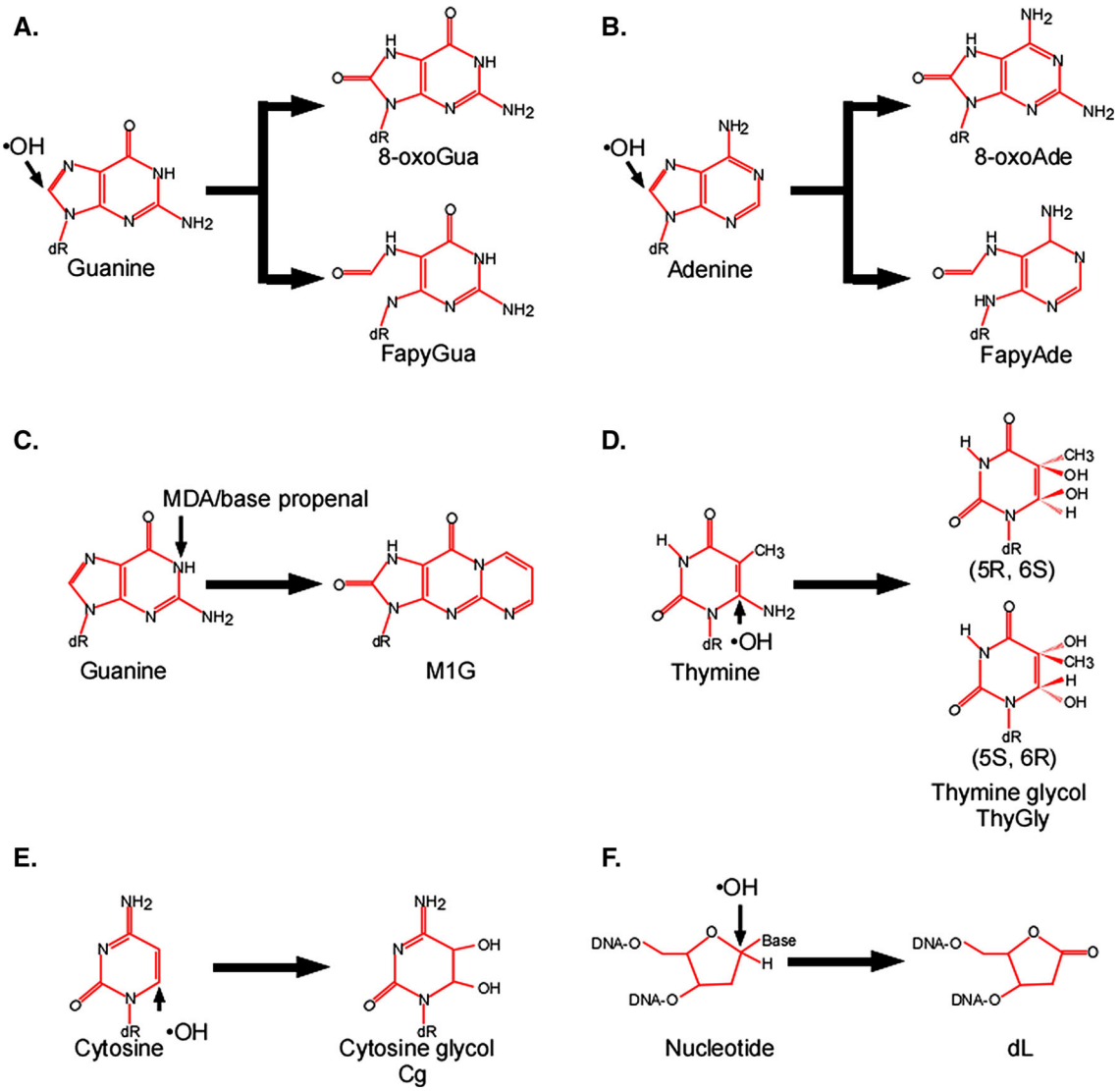


Figure 2. Formation and structure of major ODMs. 8-oxo-7,8-dihydroguanine (8-oxoGua) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) are derived from •OH addition at C8 of guanine (**A**) and the equivalent products from adenine (**B**); malondialdehyde (MDA) and the base propenal react with guanine to generate pyrimido[1,2α]purine-10(3H)one (M1G) (**C**); in thymine and cytosine, C6 are oxidized mostly by •OH and resulting in cis and trans thymine glycol (ThyGly) and cytosine glycol (CytGly), respectively (**D** and **E**). 2-deoxyribonolactone (dL) results from the C1′ hydrogen abstraction of the deoxyribose (dR), the other sugar radicals from •OH attacking at carbons leads to a strand break (**F**).

(OGG1) at the glycosydic bond between the damaged base and sugar. The OGG1-associated lyase breaks the phosphodiester backbone 3′ to an abasic site. This is followed by apurinic/apyrimidinic endonuclease cleavage of the 5′ phosphodiester bond, leading a one-nucleotide gap. This gap is filled by DNA polymerase γ, and eventually sealed by DNA ligase III (Mandavilli *et al.* 2002) (figure 3).

It has also been demonstrated that transcription-coupled DNA lesion repairing factors CSA and CSB involved in 8-oxoGua depletion (figure 3). Both of CSA and CSB localize in mitochondria and interact with mtDNA. The absence of CSA or CSB leads to increased levels of 8-oxoGua in mtDNA. Physical interaction with OGG1 and mitochondrial single-strand binding protein 1 indicates their involvement in repairing of oxidatively induced 8-oxoGua (Aamann *et al.* 2010; Kamenisch *et al.* 2010). It has been proposed that CSB influences the rate of 8-oxoGua repair by regulating OGG1 expression or activity; however, the mechanism is not clear. It is likely that CSB specifically stimulates transcription of the OGG1 gene which is supported by RT-PCR experiments (Tuo *et al.* 2002). Alternatively, CSB may interact with OGG1 and stimulate its activity in the mitochondria, which is supported by a functional crosstalk between the CSB and OGG1 proteins in cell extracts. The link between NER proteins CSA, CSB, and the BER protein OGG-1 remains unclear. However, this is suggesting the NER pathway is also implicated in oxidative mtDNA damage, playing the protective roles of mtDNA in segmental progeroid symptom of Cockayne syndrome patients, and prevents normal human aging.

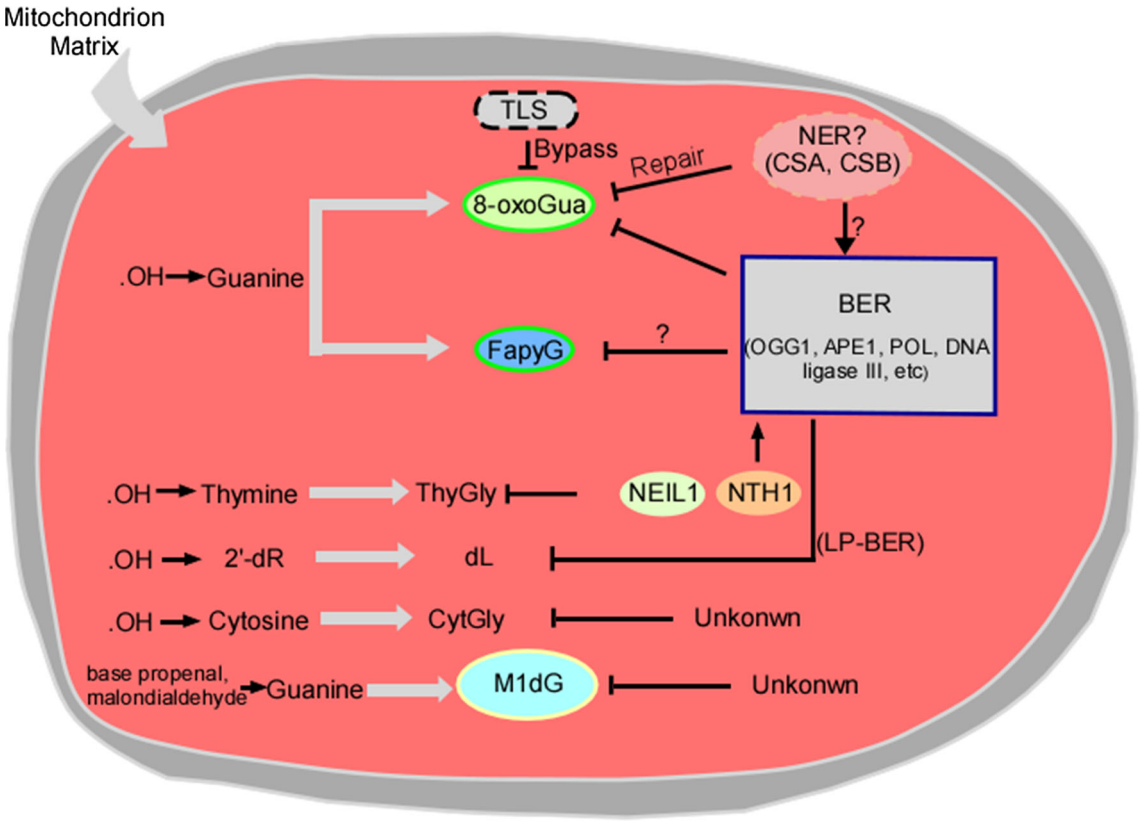
In the presence of 8-oxoGua, mitochondria employ the error-prone DNA damage tolerance pathway (TLS) to bypass DNA lesions and allow DNA replications to proceed (Waters *et al.* 2009; Sale 2013) (figure 3). In the mitochondria, DNA polymerase γ exhibits TLS activity and is able to bypass 8-oxoGua which gives rise to increased mutagenicity (Hanes *et al.* 2006). PrimPol, a DNA primasepolymerase, was also identified in the mammalian mitochondria and evidenced for its ability to bypass 8-oxoGua (García-Gómez *et al.* 2013; Guilliam *et al.* 2015). Both DNA polymerases Polζ and Rev1 have been identified in yeast mitochondria and have roles in reduction of point mutations in the mtDNA; however, it remains unknown whether they are involved in 8-oxoGua elimination (Zhang *et al.* 2005; Baruffini *et al.* 2012).

Due to the correlation between the intracellular ROS and the number of 8-oxoGua lesions in nuclear and mitochondrial DNA, 8-oxoGua has been realized as the biomarker to reflect oxidative-stress in diseases models. Under hyperoxia condition, increased perinuclear 8-oxoGua immunoreactivity has been observed in epithelial cells of the lung in mice (Roper *et al.* 2004). This phenomenon suggests the causative role of oxidative DNA modifications in lung diseases. It has also been reported that nickel exposure induces high occurrence of 8-oxoGua in mitochondrial DNA in Neuro2a cells, causing the neurotoxicity. Consistently, nickel has been found to destroy mtDNA nucleoid structure and organization. This effect can be efficiently attenuated by melatonin pretreatment. OGG1, which removes 8-oxoGua is detected with low activity in mitochondria from breast, lung and prostate cancer cell lines (Mambo *et al.* 2002, 2005; Karahalil *et al.* 2010; Trzeciak *et al.* 2004). The removal function of OGG1 has also been applied for the oxidative damage measurements. As formation of 8-oxoGua does not interfere the structure of DNA, while it exerts negligible effects on Taq DNA polymerase. However, when treated with OGG1, the 8-oxoGua-containing DNA template will be cleaved at the lesion site. As a result, the amplification process mediated by Taq DNA polymerase is disrupted and no amplicons can be detected. Thus, this method reflects damages caused by oxidative stress and has been applied for oxidative stress-related disease such as vitiligo (Vaseghi *et al.* 2017; Murphy *et al.* 2008; Wallace 2002).

3.2 *M1dG, a major peroxidation-derived DNA adduct*

Mitochondrial •OH and peroxynitrite are oxidatively reactive to convert nucleotides or lipids to base propenal and malondialdehyde, respectively, via prostaglandin synthesis and lipid peroxidation breakdown (Marnett 2000; Bigey *et al.* 1995; Plastaras *et al.* 2000). Both products are readily reactive with DNA to generate 3-(2-deoxy-β-D-erythro-pentofuranosyl) pyrimido[1,2-α]purin-10(3H)-one (M1dG), highly prevalent among all adducts (Plastaras *et al.* 2002) (figure 2C). Increased base-pair substitutions and frameshifts are correlated with M1dG occurrences. This adduct can be displaced by NER in ncuclei, however, as individual nucleotides are oxidized by oxidase from cytosol (Wauchope *et al.* 2015).

M1dG has been reported with higher level in mtDNA in comparison to nuclear DNA among a variety of human cell lines, approximately 50- to 100-fold. The M1dG scatter randomly at the mitochondrial genome, instead of gathering with sequence specificity. Its abundance is



Repairing enzyme	Activity
OGG1 (BER)	Attack of 8-oxoGua at the glycosydic bond
APE1 (BER)	Apurinic/apyrimidinic endonuclease cleavage of the 5' phosphodiester bond
POL (BER)	Fill one-nucleotide gap
NEIL1, NTH1	Excise ThyGly from the genome
CSA, CSB	Involved in 8-oxoGua depletion

Figure 3. Mitochondrial ODMs and the repairing pathways. With guanine ring attacked by •OH, intermediate adducts can be either reduced to 8-oxoGua or oxidized to yield FapyGua. Thymine glycol (ThyGly) and cytosine glycol (CytGly) are also the products from •OH oxidization on thymine and cytosine, respectively. The product of •OH attack to the 2′-deoxyribose (dR) of nucleoside is the 2-deoxyribonolactone (dL). Base propenal or malondialdehyde oxidize guanine to yield 3-(2-deoxy-ß-D-erythro-pentofuranosyl) pyrimido[1,2-a]purin-10(3H)-one (M1dG). For the repairing, BER handles small, non-distorting lesions 8-oxoGua, FapyGua and FapyAde. DNA lesion repairing factors CSA or CSB are involved in repairing 8-oxoGua in mtDNA. The link between NER proteins CSA, CSB, and the BER protein OGG-1 remain unclear. The error-prone DNA damage tolerance pathway (TLS) is employed to bypass 8-oxoGua DNA lesions and maintain the DNA replications to proceed. NTH1, Endonuclease III-like 1, initiates the BER repair of FapyGua. NEIL1, mammalian homologues of the bacterial Nei glycosylase have been identified to excise primarily FapyAde. NEIL1 and NTH1 are DNA repair enzymes specialized in excising genomic ThyGly may also repair mitochondrial ThyGly. The FEN1 mediated LP-BER pathway likely plays important roles not only in oxidative lesions including dL lesions but also in normal mtDNA replication, while the repairing pathway in mitochondria for both CytGly and M1G remain unknown.

highly correlated with superoxide level (Wauchope *et al.* 2018). Agents inducing the electron-leakage (e.g. rotenone or antimycin) can cause the accumulation of M1dG in mtDNA. On the other hand, mitoTEMPO, the mitochondria-targeted antioxidant, significantly decreased the M1dG derived from basal and rotenone oxidation. In contrast, the nuclear M1dG levels was not sensitive to any of these treatments (Dikalov 2011; Dikalova *et al.* 2010), suggesting the particular causative role of electrons leak on M1dG in mitochondria.

In mitochondria, robust BER pathways are available for repairing a number of DNA adducts, and yet earlier reports showed none of them were employed for repair M1dG (Akbari *et al.* 2008; Liu *et al.* 2008; Sykora *et al.* 2012) (figure 3). Different from nuclear DNA, the synthesis 6-oxo-M1dG during the adenine propenal treatment has not been observed in DNA of mitochondria, suggesting the lack of enzymatic pathway required for M1dG oxidation in the mitochondria. The transient existence of 6-oxo-M1dG during the flashing removal process may also lead to failure of its detection. It has been proposed that high level of M1dG will severely impair the mtDNA integrity, leading the DNA degradation. Subsequently, oxidized mtDNA will be released into the cytosol and hence cause the inflammasome activation (Nelson and Keller 2007).

It has been speculated that the persistence of M1dG adducts in mtDNA for its integrity and mitochondrial gene expression contribute to the role of mitochondrial dysfunction in diseases. Moreover, M1dG abundance in mtDNA has been revealed relevant with disorder in the bone morphogenetic protein signaling pathway. A two-fold elevation of M1dG level in mtDNA has been observed from transgenic mice with bone morphogenetic protein receptor 2 mutant compared to wild-type. These results are consistent with the hypothesis that oxidative stress and mtDNA damage may lead to mutations implicated with aging and neural diseases (Kujoth *et al.* 2007; Newsholme *et al.* 2007), while the steady levels of M1dG in mtDNA over time might enable it to serve as a biomarker for mitochondrial-oxidative-stress-related disease.

3.3 *Other modifications in the mitochondria*

The oxidative ROS-induced DNA damages include apurinic/apyrimidinic (basic) DNA sites, oxidized purines and pyrimidines, and single-strand and double-strand DNA breaks. FapyGua and 4,6-diamino-5-formamidopyrimidine (FapyAde), 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol, ThyGly) and 5,6-dihydroxy-5,6-dihydrocytosine (cytosine glycol, CytGly), and several base lesions are also caused by the interactions between hydroxyl radical with purines or pyrimidines (thymine and cytosine) at C5 or C6 of the ring (Kryston *et al.* 2011) (figure 2).

Formamidopyrimidine lesions result from the opening of the imidazole ring caused by the attack of ROS (Ignatov *et al.* 2017). The most common and biologically relevant oxidized derivatives of nucleobases include FapyGua and FapyAde. The ring-opened Fapys accumulate in mtDNA in mice lacking the OGG1 and a mammalian ortholog of *E. coli* endonuclease III (NTH1) N-methylpurine DNA glycosylase (Hu *et al.* 2005). In simian kidney cells, FapyGua was found with higher mutagenicity than 8-oxoGua (Kalam *et al.* 2006).

NTH1 initiates the BER repair of oxidized ring pyrimidine residues, including FapyGua and FapyAde. FapyAde incision activity was greatly reduced in mitochondrial extracts from NTH1 knockout mice, suggesting that the enzyme is functionally active in mitochondria. NEIL1, mammalian homologues of the bacterial Nei glycosylase, was found to localize to mouse liver mitochondria, and has recently been identified, which excise primarily FapyAde (Hu *et al.* 2005).

ThyGly, as a non-mutagenic lesion, is highly toxic and its presence shows the suppression on replication enzymes (Aller *et al.* 2007; Clark and Beardsley 1987). Also, with its frequency, ThyGly in DNA has been taken as a marker for oxidative stress (Cathcart *et al.* 1984; Adelman *et al.* 1988). Human NEIL1 and NTH1 are DNA repair enzymes specialized in excising ThyGly from the genome; however, statistically a few of the damaged bases will evade repair, and it has been shown as the effective block to DNA replication (Aspinwall *et al.* 1997; Bandaru *et al.* 2002; Hazra *et al.* 2002; Clark and Beardsley 1986; Hayes and LeClerc 1986; Ide *et al.* 1985; John *et al.* 1998). NTH1 binds C5-C6 of ring-saturated pyrimidines, and releases the lesion generating the AP site (Shapiro and Yamaguchi 1972). NTH was shown to recognize ThyGly, CytGly, and cytosine hydrate that are formed under oxidative stress (Ignatov *et al.* 2017).

In the DNA, only the highly reactive •OH is able to attack the 2′-deoxyribose residue and the C1′ hydrogen atom abstraction gives rise to the 2-deoxyribonolactone (dL) (Cadet *et al.* 2010). dL inhibits replication by interacting with polymerase γ during the dL excision and interferes with the removal of the DNA lesion and the incorporation of a single nucleotide, the short-patch BER. Flap endonuclease 1 (FEN1)-dependent long-patch BER has been reported to resolve dL lesions. The

FEN1-mediated long-patch BER pathway plays important roles not only in oxidative lesions including dL lesions but also in normal mtDNA replication (Hamon *et al.* 2015). Figure 3 depicts common types of ODMs with their repairing pathways.

4. Oxidized mitochondrial DNA induces mitochondrial dysfunction, nuclear response, and inflammasome activity

Mitochondrial DNA variants at nt 10398 (G10398A) and 16189 (T16189C) were associated to risk for breast cancer and endometrial cancer, respectively (Brandon *et al.* 2006). The T8993G mutation causes a L156R amino acid substitution resulting in a 70% reduction in ATP synthase (complex V) activity (Trounce *et al.* 1994). It is assumable that the ROS-induced mtDNA mutation causes a decrease in mitochondrial activity and promotes a switch to glycolysis dependent metabolism, contributing to tumorigenicity. However, the example in PGC1α-positive mutation melanoma cells exhibiting increased mitochondrial energy metabolism and ROS detoxification capacities is inconsistent with the explanation (Vazquez *et al.* 2013).

In numerous cancers, mtDNA mutations, deletions, and loss of copy number causing defective ETC complexes have been observed: (1) In breast cancer, among 18.5% of the mutations were detected in the 16S rRNA, ND2, and ATPase 6 genes, 42% were deletions or insertions. The remaining seven mutations (58%) were single-base substitutions in the coding or non-coding regions (D-loop) of the mitochondrial genome (Tan *et al.* 2002). (2) In malignant gliomas, the copy number of mtDNA were revealed when compared to a normal brain control (Liang 1996; Liang and Hays 1996; Chatterjee *et al.* 2006). The causal role of these mitochondrial defects in tumor formation has not been established. It has been demonstrated that mtDNA mutations of Complex I, III, and V subunits can induce tumorigenic transformations (Sharma *et al.* 2011; Ishikawa *et al.* 2008).

The mutation may lead to a decrease in mitochondrial mRNA and mitochondrial protein synthesis and with the subsequent electron transport disruption. Even though limited effects might be caused on the oxidative phosphorylation, it is likely to drive shifts in ETC redox status and ROS generation. For instance, mutations detrimental to the activity of Complex IV will impair the balance of redox state of complexes in ETC for electron transfer, enhancing superoxide from Complex I, II or III (Shidara *et al.* 2005). The resulting disproportionate ROS generated further disrupts cytosolic redox signaling during regulation of tumorigenesis. These have been described in a correlation study between mtDNA instability and tumorigenesis induced by increases in mitochondrial ROS generation in $APC^{Min/+}$ mouse model (Woo *et al.* 2012). The increasing generation of ROS and more mtDNA damage will be in a feed-forward cascade. Eventually the membrane potential of mitochondria collapse, resulting in the free cytochrome c in the cytoplasm and initiation of apoptosis by the activation of caspases.

While the nucleus encodes mitochondrial proteins and drives mtDNA replication and maintenance, the redox imbalance and mtDNA damage in the mitochondrion trigger signals impacting nuclear gene expression response (Jazwinski 2014). Change in Ca2+ efflux as a result of mtDNA depletion, for example, can directly influence activation of c-Jun N-terminal kinase (JNK) pathway linked to the DNA damage response, though the influence on mitochonrial DNA repair remain unclear (Biswas *et al.* 1999; Amuthan *et al.* 2001; Picco and Pages 2013; Prakash *et al.* 2016). NADH/NAD+ is an essential metabolite and coenzyme in the regulation of the ETC, and the TCA cycle in the mitochondrion (Ying 2008; Stein and Imai 2012; Canto *et al.* 2015). NAD+ as a substrate for Poly (ADP-ribose) polymerase (PARP) family, therefore, is critical for BER (Shaughnessy *et al.* 2014; Osborne *et al.* 2016; Yu and Auwerx 2009; Burkle 2005). PARP1, as a member, is reported to have an inhibitory role in the mitochondria where it inhibits BER, contrary to its function depicted in the nucleus (Szczesny *et al.* 2014).

High level of ROS has been described as an example of signaling between the mitochondria and nucleus. As a result, expression of over 100 target genes such as glutathione S-transferases mediated by the binding of NRF2 to antioxidant response elements have been stimulated (Itoh *et al.* 2015). Furthermore, the triggering of TFAM expression for driving mitochondrial transcription by NRF1 occurs in response to increased levels of ROS (Piantadosi and Suliman 2006).

Oxidized mitochondrial DNA also induces NOD, LRR, and Pyrin domain-containing protein 3 (NLRP3) inflammasome activity, an innate immune response to ROS-mediated oxidative injury. This is usually followed with the release of oxidized mtDNA into the cytosol to activate NLRP3 and NLR family CARD domain-containing protein 4 (NLRC4). The oxidized mtDNA in the cytosol maintain the stability with the binding of NLRP3, and implicated with the inflammasome activation (Nelson and Keller 2007). The

deoxyriboside form of 8-oxoGua, 8-oxo-2′-deoxyguanosine, is one of the predominate oxidative adducts in mtDNA. It increased dramatically under oxidative stress and showed the punctate staining pattern in cytoplasm, indicating the structural pattern of mitochondria, while the nuclear 8-oxo-2′-deoxyguanosine staining was homogenous. This is usually accompanied with stimulated production of Lys-63-specific deubiquitinase BRCC36 in cytoplasm of cells. BRCC36 is a critical regulator of NLRP3 deubiquitination and inflammasome activation and involved in DNA damage response at double-strand break sites (Ne Dicte *et al.* 2013; Ng *et al.* 2016). The punctate mitochondrial pattern of BRCC36 suggested that mtDNA oxidative damage may stimulate BRCC36 activity to priming and activate NLRP3 inflammasome, an innate immune response to environmental stress via ROS-mediated oxidative injury (Gong *et al.* 2018).

It is intriguing how mtDNA is delivered to the cytosol. Previous research based on Nlrp3$^{-/-}$ macrophages described the mitochondrial damage as the downstream of NLRP3 signaling (Heid *et al.* 2013). Also, mitochondrial damage and mtROS have been demonstrated not required for the NLRP3 activation, as either NEK7 or ionic flux in the cytosol seems sufficient to activate the NLRP3 (Muñoz-Planillo *et al.* 2013; Allam *et al.* 2014). A recent study suggested that mtDNA, instead of being released into the cytosol during inflammasome-mediated cell death, is transported to the cytosol prior to NLRP3 inflammasome activation (Zhong *et al.* 2018; Coll *et al.* 2018). Whether the recognition of oxidized DNA by NLRP3 is associated with other activation mechanism remains unclear.

5. Characterization of mitochondrial ODMs in pathological conditions

Mitochondrial dysfunction is one of the key hallmarks of aging process and is implicated in the numerous age-related pathologies including cardiovascular diseases, metabolic syndrome, diabetes, obesity, neurodegenerative disorders (Alzheimer's, Parkinson's, and Hungtinton's diseases), and cancer (Lemieux and Hoppel 2009; Gurzov and Eizirik 2011; Lin and Beal 2006; Chandra and Singh 2011; Bohr and de Souza-Pinto 2010). In all these diseases, oxidative stress, increased ROS production, and accumulation of ODMs have been postulated contributing to mitochondrial dysfunction, and accurate measurements of mitochondrial ODMs are required to validate the possible correlation between ODMs number and the dysfunctional state of the mitochondria (Fernandez-Checa *et al.* 2010; Halestrap and Pasdois 2009; Wanagat *et al.* 2001).

Mitochondrial ODMs measurements remain the primary challenge for characterizing ODMs' impacts on mitochondria dysfunction-related diseases. Quality control of DNA sample and the artefacts interference of measuring methods are major issues. Initially, quantification of ODMs such as 8-oxoGua was measure by technique high-performance liquid chromatography with electrochemical detection (HPLC-ECD) on nucleosides hydrolyzed from DNA. However, even with the European Standards Committee on Oxidative DNA Damage established, inconsistent measuring results from different labs remain, due to artefact oxidation during DNA isolation and sample preparation (Richter *et al.* 1988; ESCODD 2002). Moreover, 8-oxoGua as a biomarker by HPLC-ECD may not always reflect the level of DNA damages because of the redox-status-dependent generation of the 8-oxoGua and FapyGua, causing ratio variation of omitted FapyGua.

With the advantage of structural evidence for an analyte and accurate quantification (MS with isotope dilution), GC-MS detection has been employed to analyze the levels of oxidized nucleobases. Bases such as 8-oxoGua, FapyGua, dT, etc., can be measured by GC-MS, following the enzymatic hydrolysis of DNA (Dizdaroglu *et al.* 2002; Dizdaroglu 1986, 2012). Still, artefacts occur during GC-MS measurements. The oxidation of intact DNA bases introduced during the high-temperature derivatization, e.g. guanine converted into 8-oxoGua (Cadet *et al.* 1997, 2010; Ravanat *et al.* 1995; Douki *et al.* 1996). The exclusion of oxygen with pure nitrogen and derivatization at room have been found to prevent the oxidation of guanine to 8-oxoGua.

An HPLC-based purification process has been applied to remove intact DNA bases via hydrolysis and derivatization. The development of HPLC coupled to tandem mass spectrometry has made it possible to accurately detect modifications in cellular DNA. The availability of capillary liquid chromatography associated with nano-electrospray ionization tandem mass spectrometry (LC-NSI-MS/MS) demonstrates a significant increase in the sensitivity of detection. However, when applied to mitochondrial DNA, this method is also limited by the accuracy on low amounts of nucleic acids measurements. The highly varying values of 8-oxoGua within the range of 130–400 lesions/10^6 are possibly due to the occurrence of artefactual oxidation. Moreover, without the exclusion of air during

derivatization, artefactual formation of 8-oxoGua would be introduced (Ravanat *et al.* 1995; Douki *et al.* 1996; Cadet *et al.* 1997). The destructive effects on both FapyAde and FapyGua during DNA hydrolysis with formic acid (Cadet *et al.* 1997; Douki *et al.* 1997) and tedious procedures also compromise the prevalent applications for ODMs measurements. So far there has not been accurate documentation on the steady-state levels of 8-oxoGua in mitochondrial DNA (Ma *et al.* 2016; Yu *et al.* 2018).

Fluorescent-labeled antibody of 8-oxoGua has been used to demonstrate the increased cytoplasmic immunoreactivity on igrostriatal dopaminergic neurons of Parkinson's disease patients and mouse model (Shimura-Miura *et al.* 1999). However, the specificity of antibodies to detect oxidized bases in the mitochondria remain to be verified.

6. Conclusions and future perspective

With the single electron reduction of O_2, the superoxide will be produced in mitochondria. As stated above, spontaneous and superoxide dismutase (SOD)-dependent O2•− dismutation produces H_2O_2, which can be transformed into •OH by the Fenton reaction with the availability of transition metals. The oxidation with nucleic acids, lipids and proteins escalates along this reaction cascade and is topped by •OH (Valko *et al.* 2007; Moreira 2012). All four bases (adenine, guanine, cytosine, thymine) and the corresponding deoxynucleosides are highly susceptible to •OH attacking. As a result, there are 20 oxidized ODMs produced and identified in the mitochondria (Cooke *et al.* 2003).

ODMs are more frequently produced in some pathological conditions, particularly in cancers and neurodegeneration. The linking between ODMs such as 8-oxoGua and pathological conditions qualify its application as extensive biomarkers. Nonetheless, quantification of ODMs have been the main challenge for the correlations between ODMs profile and pathological conditions due to artefacts introduced during the measurement. The development of artefact-reduced technique to quantify 8-oxoGua lesions and other modifications (Garcia *et al.* 2010). Along single-cell sequencing based mutational analysis (Dong *et al.* 2017; Ludwig *et al.* 2019), the scope of ODMs mapping within the mitochondria will be broadened. All these will be important to deepen the understanding of the molecular events that manifests in diseases that impact public health, e.g. obesity, diabetes, and cardiovascular disease. Further, comparison of these mitochondrial ODMs from different tissues and at different stage of development will reveal the effects brought by these modifications at cellular level and tissue level and their specificity in different tissues (Muftuoglu *et al.* 2014).

For most ODMs generated in the mitochondria, BER is the major pathway to repair mitochondrial ODMs and has been comprehensively characterized. NER proteins CSA and CSB were reported to involved in 8-oxoGua depletion, also XPD derived from xeroderma pigmentosum was observed to provide protection for mtDNA from oxidative damage. Nevertheless, the existence of canonical NER pathway in mitochondria are not fully supported by experimental data. Global genomic repair (GGR) and bacterial transcription coupled DNA repair (TCR) are identified as two divergent NER pathways, with the latter initiated by the stalled RNA polymerase (RNAP) at the DNA lesions in its path (Kamarthapu and Nudler 2015; Bohr *et al.* 1985; Mellon and Hanawalt 1989; Tornaletti and Hanawalt 1999). The recruitments NER enzymes or even BER enzymes to the DNA lesion by single-subunit RNA polymerase (mtRNAP) has not been ruled out. Presence of TLS activity in mitochondria was exhibited by DNA polymerase γ and PrimPol to maintain the fidelity of mtDNA during replication. To identify the repairing manner of both NER and TLS in mitochondria, relevant data need to be further clarified and substantiated. The regulation role of PARP1 in nuclear DNA transactions is via nuclear DNA single-strand breaks recognizing and the recruitment of DNA repair enzymes to the site (Haince *et al.* 2008; de Murcia *et al.* 1997; Trucco *et al.* 1998). In the mitochondria, though PARP1 has been reported to interact with 5′-exo/endonuclease and DNA polymerase γ, the key mitochondrial BER enzymes, its presence compromises the coordination of BER pathway. Under the oxidative-induced mitochondrial damage condition, the abolish of PARP1 not only elevates the repair activity of BER, but also enhances mitochondrial DNA copy number and mitochondrial membrane potential. For mitochondrial biogenesis and energy metabolism regulators, e.g. peroxisome proliferator-activated receptor γ coactivator 1α, which can upregulate the ROS-detoxifying enzymes level under the oxidative stress (St-Pierre *et al.* 2006), may exert certain effects over DNA repair in mitochondria. In sum, repairing pathways in mitochondria are rather complexing and remain unclear, with more functional protein characterized, the coordinating manner among pathways will be unveiled.

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