

**Oxidized SOD1 accelerates aging by compromising the formation of
mitochondrial-derived vesicles**

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Abstract

Aging is a complex process marked by a gradual decline in the function of multiple organelles, contributing to various age-related disorders. The specific causes and underlying mechanisms of aging remain largely unknown.

Superoxide dismutase 1 (SOD1) is an antioxidant but is susceptible to oxidative modification. Once oxidized, SOD1 undergoes aberrant misfolding and acquires toxic properties associated with age-related disorders. The present study aims to investigate the role of oxidized SOD1 in aging. We found that the mouse motor neuron-like NSC34 cells expressing the human *SOD1* (hSOD1) gene with a *G93A* mutation (*hSOD1*^{G93A}) underwent early entrance into senescence, and the *hSOD1*^{G93A} transgenic mice exhibited aging phenotypes at an early age. In addition, we discovered that the wild-type hSOD1, when oxidized, could also induce cellular senescence in neural stem cells (NSCs). A high level of oxidized SOD1 was present in both senescent NSC34 cells and mouse NSCs expressing the *hSOD1* gene. Notably, the extracellular vesicles (EVs) derived from senescent NSCs containing a high level of oxidized SOD1 accelerated the senescence of young NSCs and induced the death of neurons in the culture. Blocking oxidized SOD1 in EVs by an interference peptide restored the proliferation of NSCs and mitigated the toxicity of EVs to neurons. Additionally, we found mitochondria malfunctioned in both senescent NSC34 cells and mouse motor neurons of *hSOD1*^{G93A} transgenic mice in middle age. Interestingly, mitochondria exhibited increased production of mitochondrial-derived vesicles (MDVs) during the early stages of aging; however, the responsiveness of mitochondria to mild oxidative stress gradually declined.

In conclusion, our data show that oxidized SOD1 increases in senescent cells and transgenic mice with advancing age. Targeted removal of oxidized SOD1 significantly decreases biomarkers of

senescence. Therefore, oxidized SOD1 is a causal factor of aging. Selective removal of oxidized SOD1 represents a promising strategy to rejuvenate senescent NSCs and decelerate aging. Meanwhile, the modulation of MDVs plays a role in early aging. Thus, the mitochondrial responsiveness to produce MDVs could serve as an indicator of premature aging.

Keywords: Aging, Cellular senescence, Superoxide dismutase 1, Oxidative modification, Mitochondrial dysfunction, Mitochondrial-derived vesicles

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Dedication

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Chapter I Introduction

1. Introduction

Aging is characterized by a decline in biological and metabolic activity, leading to various age-associated disorders, such as neurodegenerative diseases, cardiovascular diseases, and cancer. Over the past two decades, significant efforts have been directed towards identifying age-associated factors to enhance our comprehension of the aging process and discover interventions that can enhance human longevity and enhance the quality of life.

Aging organisms are characterised by the development of cellular senescence, a condition of permanent cell cycle arrest triggered by various harmful events. When senescent cells accumulate in tissues, it may hinder the body's natural ability to repair itself and foster an inflammatory environment that promotes the development and spread of age-related disorders like neurodegenerative diseases. Yet, senescent cells serve several vital roles in the body. Cellular senescence is a potent defence mechanism against cancer because it causes an irreversible proliferative halt. Moreover, senescent cells may positively regulate tissue remodelling and repair at various stages of development and maturity through cell and non-cell autonomous pathways. Prominent hallmarks of the senescence phenotype include DNA damage, cell cycle arrest, increased lysosome activity, senescence-associated secretory phenotype, changes in metabolic rate, endoplasmic reticulum stress, and stimulation of antiapoptotic genes, modified nucleus, enlarged and irregular cellular shape, and increased number of lysosomes and mitochondria.

Mitochondria play a crucial role in facilitating essential energy conversion processes within cells. Additionally, they serve as significant intracellular sources of reactive oxygen species (ROS) generated during oxidative phosphorylation. Beyond their involvement in intrinsic cell signaling pathways, ROS also contribute to processes linked to accelerated aging. Mitochondrial quality

control (MQC) mechanisms work to maintain relatively low and stable ROS levels. These mechanisms involve the removal of damaged mitochondrial components through various processes such as mitochondrial proteases, the mitochondrial unfolded protein response (UPR^{mt}), mitochondrial-derived vesicles (MDVs), and mitochondrial autophagy (mitophagy). At the molecular level, one aspect of MQC is the presence of mitochondrial proteases across different mitochondrial compartments. These proteases perform several proteolytic functions within MQC, including the elimination of damaged or misfolded polypeptides, regulation of protein processing, maturation cleavage, and protein assembly¹{Jin, 2013 #1322}. UPR^{mt} facilitates the refolding and degradation of misfolded proteins within mitochondria². Additionally, MDVs and mitophagy play a role in partially or completely removing damaged mitochondria along with oxidized by-products³. Moreover, a complex signaling network is involved in coordinating the aforementioned mitochondrial quality control processes⁴.

Dysfunctional mitochondria can contribute to elevated reactive oxygen species (ROS) production, thereby accelerating the aging process. Additionally, oxidative damage to mitochondrial DNA, proteins, and lipids can further enhance ROS production during aging. Therefore, maintaining uninterrupted MQC is crucial to effectively mitigate and clear such damages. Therefore, it is imperative to prioritize the exploration of the underlying mechanisms governing MQC. Such research endeavors hold the potential to unlock novel avenues for developing therapeutic interventions that specifically target MQC pathways. By doing so, we may effectively extend the overall health span and enhance the quality of life in individuals as they age.

1.1 Cellular senescence and aging

Aging is a time-dependent functional decline that leads to a high risk of diseases and death⁵. During aging, our bodies undergo irreversible and readily identifiable changes in their appearance

and functions ⁶. Nine significant hallmarks of aging have been identified as biomarkers of aging ⁷. Cellular senescence is one of the many features of aged organisms ⁸. Cells undergo senescence due to various intrinsic and extrinsic stimuli ^{9,10}. The intrinsic factors include chromatin disorganization, telomere shortening, oncogenic activation, epigenetic activation, and perturbed proteostasis.

In contrast, the extrinsic stimuli include radiation, inflammation, irradiation, chemotherapeutic agents, oxidative stress, genotoxic stress, and nutrient deprivation ⁹. When cells come into permanent cell cycle arrest, they may have dramatic secretory phenotypes ¹¹, mitochondrial dysfunctions ¹², and morphologic changes ¹³. Interestingly, these cells remain metabolically active and secrete many factors, including growth factors, cytokines, and chemokines (known as senescence-associated secretory phenotype, SASP) ¹⁴. However, these changes hinder the capacity to repair and replace damaged tissues, a key factor in aging ¹⁵. Evidence confirms that suppressing the growth of cells in response to tissue dysfunction associated with aging has been shown to increase longevity ¹⁶.

1.1.1 Permanent cell cycle arrest

The precise regulation of cellular proliferation is essential for tissue homeostasis and development, whereas improper regulation results in diseases ¹⁷. Different modes of growth arrest, such as quiescence and senescence, are used by cells for fine-tuned regulation ¹⁸. Quiescence is a non-proliferative condition generated by many factors, such as mitogen deprivation, contact inhibition, and adhesion loss ¹⁹. The capability of cells to re-enter the cell cycle after reversing these factors is a hallmark of quiescence ²⁰. In contrast to quiescence, senescence is an irreversible growth arrest ²¹. Senescent cells continue to have a high metabolism, unlike quiescent cells, which have a low metabolism ²².

Additionally, the decision to enter which state of growth arrest is mediated by the core cell-cycle machinery in response to various external and internal signals. There are typically four phases to a normal cell cycle: G1, S, G2, and M for mitosis. Most cells that aren't dividing leave the cell cycle and enter a quiescent state (G0), from which they can re-enter the process if stimulated. The positive regulation of the cell cycle is regulated by two classes of proteins termed cyclins and cyclin-dependent kinases (CDKs). Cyclins only attaching to CDKs exert their regulatory influence on the cell cycle. Besides, the CDK/cyclin complex must be phosphorylated to be fully active and able to phosphorylate other proteins that progress the cell cycle ²³. The CDK inhibitors (CDKIs) encoded by the CDKN2A (p16^{INK4a}, p16), CDKN2B (p15^{INK4b}, p15), and CDKN1A (p21^{CIP}, p21) are the primary regulators of cell cycle arrest ²⁴. Quiescent cells use CIP/KIP inhibitors to bind to and inhibit CDK/cyclin complexes, whereas senescent cells depend on p16 to directly inhibit CDK4 and CDK6 ²⁵. The transcriptional activation of p16 has been widely employed to report the presence of senescent cells in vivo since it is a highly specific and novel marker for senescence ²⁶. While cyclins are the primary regulatory molecules responsible for propelling the cell cycle forward, other mechanisms negatively fine-tune the cycle's progression. These mechanisms work to halt the cell cycle until problematic conditions are resolved. CDKIs are molecules that inhibit the full activation of CDKs. They are known to monitor specific cell cycle events either directly or indirectly. The block imposed on CDKs by inhibitor molecules persists until the monitoring event is completed. Negative regulators have been extensively studied, including retinoblastoma protein (Rb), p53, and p21. Rb, p53, and p21 are essential regulators of the G1 checkpoint. Rb regulates positive regulator proteins and monitors cell size, interacting with E2F and other transcription factors in its active, dephosphorylated state. Transcription factors activated by Rb promote the production of specific proteins necessary for the G1/S transition. CDKs typically

phosphorylate Rb to promote the transition from G1 to S phase²⁷. Some essential proteins involved in cell cycle progression are phosphorylated or ubiquitinated^{28,29}. When Rb binds to E2F, the transition protein genes are not expressed. Rb becomes increasingly phosphorylated as the cell grows, resulting in its inactivation. Once Rb is inactivated, E2F is released and can activate the gene encoding the transition protein³⁰. p53, a multifunctional protein, is vital in determining whether a cell will divide. It activates when cells undergoing G1-phase preparations detect DNA damage. p53 halts the cell cycle and summons enzymes to repair any DNA damage. However, if the DNA is damaged beyond repair, p53 triggers senescence, apoptosis, or programmed cell death³¹⁻³³. When p53 levels increase, p21 expression is stimulated. The p21 protein serves as a broad CDKIs, which typically phosphorylates Rb to promote the transition from G1 to the S phase. p21 prevents the activity of CDK/cyclin complexes by binding to them, thereby enforcing the cycle arrest directed by p53³⁴. All positive regulators must be activated to reach each checkpoint, while all negative regulators must be inhibited³⁵. When cells come to the senescent state, the rise of p53 and p21 levels reduces the likelihood of a cell entering the S phase, leading to permanent cell cycle arrest. When activated, p53 promotes the expression of p21^{CIP1/WAF1} and inhibits cell cycle proteins cyclin A, E, and D. Additionally, p16^{INK4a} can activate the pRB pathway independent of p53. This occurs because p16^{INK4a} blocks the activity of kinase complexes such as Cyclin A, Cyclin E, and Cyclin D-dependent kinases, which would otherwise phosphorylate and inactivate pRB. The dephosphorylated pRB inhibits the G1/S transition by sequestering E2F transcription factors and suppressing E2F-dependent gene expression³⁶. Studies offer additional support for the efficacy of a highly specific CDK4/6 inhibitor in combatting a subset of neuroblastoma cell lines and xenograft models, underscoring the need for further research into its potential as a treatment option for individuals with this type of cancer³⁷. The presence of p16 and p21, particularly in astrocytes,

indicates the potential induction of senescence in glial cells. Thus, increased expression of p16 and p21 in the frontal association cortex of donors with amyotrophic lateral sclerosis/motor neuron disease (ALS/MND) may be an early indication of senescence activation and cell cycle dysregulation.³⁸

1.1.2 Activated metabolism and morphological changes

The senescent phenotype encompasses more than just a disruption in cell growth. Studies have shown that senescent cells exhibit metabolic activity, as indicated by increased AMP:ATP and ADP:ATP ratios³⁹. AMP-activated protein kinase (AMPK) is allosterically activated by AMP, which prevents its dephosphorylation, making it a sensor of decreased energy levels. AMPK regulates p53 and other targets, boosting catabolic pathways while inhibiting biosynthetic ones. p53, in turn, regulates cellular metabolism by inhibiting glucose uptake and glycolysis while promoting the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and fatty-acid oxidation, which ultimately leads to the suppression of tumours by senescent cells with high TCA cycle activity⁴⁰. Mitochondrial dysfunction and mTOR activation also contribute to metabolic changes⁴¹. During senescence, mitochondrial dysfunction-associated senescence activates AMPK, which, in turn, decreases the TCA cycle and Nicotinamide Adenine Dinucleotide (NAD⁺)⁴².

Senescent cells are characterized by an expanded and atypically formed cell body⁴³. Upon exposure to stimuli that induce senescence, mTORC1 is activated, as it integrates multiple stress signals and regulates cell growth accordingly. Reduction in growth factors such as GDF11, which occurs during aging, may lead to mTORC1 activation and hypertrophy. The post-translational mechanisms that regulate mTORC1 include activation of its catalytic site by the GTPase Rheb and a subsequent rearrangement of the whole mTORC1 complex that enhances interaction with

downstream effectors ⁴³. Senescence also involves the rearrangement of the cytoskeleton, particularly the actin and vimentin filaments, contributing to deformed cellular appearance ⁴⁴. Several lysosomal proteins are upregulated, and lysosomal content is elevated in the senescent state ⁴⁵. Increased lysosomal content in senescent cells is generally measured by the lysosomal enzyme senescence-associated beta-galactosidase (SA- β -gal) activity. The *GLB1* gene codes for many transcripts, including the SA- β -gal ⁴⁶. The accumulation of aged lysosomes or increased lysosomal biogenesis may contribute to the increased lysosomal content in senescent cells ⁴⁷. The absence of lysosomal clearance is supported by the presence of residual bodies, particularly lipofuscins. The transcription factor EB (TFEB) is a key regulator of lysosomal biogenesis because it acts as a downstream effector of the mTOR signalling pathway and coordinately regulates several lysosomal proteins. In response to oncogene-induced senescence, TFEB has been found to bind the promoter of another lysosomal enzyme, β -hexosaminidase ⁴⁸. Nonetheless, there have been suggestions that TFEB may be either upregulated or downregulated during senescence or aging, which raises questions about its suitability as an indicator of senescence ⁴⁹. Besides, high lysosomal activity is not a reliable indicator of cellular senescence as well, because no senescent cells have been shown to produce SA- β -gal consistently ⁵⁰.

1.1.3 Senescence-associated secretory phenotype (SASP)

Due to being metabolically active, senescent cells have experience⁵¹d extensive protein expression and secretion alterations and can secrete bioactive molecules, known as the senescence-associated secretory phenotype (SASP). The SASP is typically thought of as proinflammatory, and a range of cytokines, growth factors, and extracellular proteases play a role in how it affects the microenvironment. The inflammatory transcription factor NF- κ B regulates the SASP transcriptional programme ⁵². Secretory phenotype markers include cytokines (interleukin-1 α , IL-

6, and IL-8), chemokines (chemokine ligand 2), and metalloproteinases (matrix metalloproteinase-1 and-3). Several factors, especially IL-6, are often measured by their protein expression and secretion using immunohistochemistry or enzyme-linked immunosorbent assay. Various cell types exhibit distinct compositions of the SASP and can engage in diverse senescence programs, including replicative senescence, DNA damage-induced senescence, or oncogene-induced senescence. The effects of the SASP can vary depending on the situations ⁵³. Take the tumors for example: on the one hand, SASP can halt cell division and stimulate the immune system to eliminate damaged cells, thereby inhibiting tumor growth. On the other hand, SASP can create an immunosuppressive environment that fosters the development and recurrence of tumors ⁵⁴.

1.1.4 DNA damage and protein aggregation

In recent years, mounting evidence suggests that DNA damage plays a crucial role in both replicative and premature cellular senescence ⁵⁵. Studies have highlighted a decline in DNA repair capability and an increase in ROS production as potential reasons for DNA damage accumulation in old age ⁵⁶. Premature aging is often caused by genetic mutations or altered gene expression that promote DNA damage. Genetic information is constantly being altered by a variety of stimuli, leading to mutations in genomic structure and DNA damage ⁵⁷. Unrepaired DNA double-strand breaks can result in mutations or chromosomal rearrangements if they remain unrepaired before cell division. Nevertheless, therapies that boost the body's defences against oxidative stress and minimize the impact of DNA damage can positively affect lifespan ⁵⁸. The available data support genomic instability as a leading factor driving the aging process ⁵⁹. However, research indicates that the formation of ROS and the extent of oxidative damage to macromolecules like DNA do not necessarily correlate with longevity in model animals ⁶⁰.

Protein aggregation refers to the process in which proteins misfold and form insoluble aggregates that can accumulate in cells and tissues ⁶¹. This process is linked to age-related diseases such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis and Huntington's disease ⁶². Recent research indicates that protein aggregation may be associated with cellular senescence ⁶³. Studies show that proteotoxic stressors and senescence markers decreased following interventions like treatment with an mTOR antagonist, a protein synthesis inhibitor, or a chemical agent to reduce the number of protein aggregates ⁶⁴. These findings suggest that targeting these pathways could be a promising therapeutic strategy for mitigating the adverse effects of senescence in neurons. Moreover, studies have shown that protein aggregates induce cellular senescence, and senescent cells can accumulate protein aggregates ⁶⁵. This could be due, in part, to the fact that protein aggregation can cause cellular stress and damage, which can trigger senescence pathways. Furthermore, senescent cells may have a reduced capacity to clear protein aggregates, leading to their accumulation ⁶⁶. The relationship between protein aggregation and cellular senescence is complex and not fully understood. Nevertheless, this area of research presents an opportunity for developing new therapies for age-related diseases. Targeting protein aggregation or senescence pathways may aid in preventing or slowing the aging progression ⁶⁷.

1.2 Mitochondrial Function and Aging

Aging is a complex process that involves a decline in biological and metabolic activity, resulting in the onset of various age-related conditions, such as neurodegenerative diseases, cardiovascular diseases, metabolic diseases, immune system diseases, and cancer ⁵⁷. The past two decades have witnessed a wealth of promising work on age-associated factors that help us better understand aging and uncover interventions that can increase human longevity and improve quality of life ⁶⁸⁻

⁷⁰.

One key aspect of aging that has been extensively studied is its impact on mitochondrial function. Mitochondria facilitate the fundamental energy conversion processes within cells ⁷¹. In addition, they are the primary intracellular source of ROS generated during oxidative phosphorylation ⁷². It is now generally recognized that ROS plays crucial roles in cellular differentiation and homeostasis ⁷³. In addition, each ROS has its own set of characteristics. Highly reactive species, such as hydroxyl radical or peroxynitrite, may initiate lipid peroxidation when they react with membrane lipids and destroy various kinds of biological molecules, resulting in more complicated radicals and severe oxidative stress. On the other hand, less reactive species, including superoxide and hydrogen peroxide, contribute to cellular signalling ^{74,75}. Apart from participating in intrinsic cell signalling pathways, ROS contributes to processes associated with aging ⁷⁶. While increased levels of mitochondrial ROS may not be the direct cause of aging according to existing evidence ⁷⁷⁻⁸⁰, they do contribute significantly to the development of age-related diseases when combined with other factors, such as epigenetic alterations or a decrease in quality control systems ⁸¹⁻⁸³. In addition, mitochondrial activity may not decline linearly throughout the aging process ⁸⁴. Research suggests that middle-aged individuals experience increased mitochondrial ROS as a signal to activate protective systems ⁸⁵. However, persistent induction of ROS can lead to oxidative damage in old age ⁸⁶. As a result, ROS has the potential to accelerate the onset of aging and diminishes the quality of life in senior individuals ⁸¹.

MQC keeps ROS relatively low and steady ⁸⁷. It disposes of the damaged part of mitochondria through mitochondrial proteases, mitochondrial unfolded protein response (UPR^{mt}), MDVs, and mitophagy. Mitochondrial proteases perform numerous proteolytic tasks through MQC, including the removal of damaged or misfolded polypeptides, regulation of protein processing, maturation cleavage, and assembling ⁸⁸. UPR^{mt} enhances misfolded protein refolding and degradation in

mitochondria ⁸⁹. Meanwhile, MDVs and mitophagy partially or entirely dispose of the damaged mitochondria and oxidized by-products ⁹⁰. A complex signalling network is involved in the above mitochondrial quality control ⁴.

Aging causes a decrease in the efficiency of oxidative phosphorylation, leading to alterations in ATP production and an increase in ROS production ⁹¹. This increased ROS production can lead to the oxidization of mitochondrial DNA, proteins, and lipids, which further reduces mitochondrial dynamics and impedes mitophagy, ultimately resulting in mitochondrial dysfunction ^{92,93}. This creates a vicious cycle, as dysfunctional mitochondria produce even greater ROS, further decreasing mitochondrial biogenesis and increasing mitochondrial damage ⁹⁴. While this cycle is closely related to aging, a thorough understanding of the mechanisms underlying MQC may help identify potential therapeutic targets to extend the overall healthy lifespan ⁹⁵.

1.2.1 Mitochondrial function and biogenesis

Mitochondria are membrane-bound organelles, constituting one-fifth of the cellular volume in most eukaryotic cells. Each mitochondrion has outer and inner membranes. Large molecules and ions are primarily permeable to the outer mitochondrial membrane (OMM) but hardly permeable to the inner mitochondrial membrane (IMM) ⁹⁶. Mitochondria are crucial in cellular metabolism and responsible for producing ATP. An electrochemical potential gradient across the IMM harbours the electron transport chain (ETC) to synthesize ATP through oxidative phosphorylation. Apart from energy metabolism, mitochondria are also responsible for other activities, such as heat generation, calcium and iron exchange, heme biosynthesis, cellular differentiation, proliferation, apoptosis, and cell death ⁹⁷.

Mitochondrial biogenesis takes place in healthy cells with a complex and multistep process. Induction of mitochondrial biogenesis involves activating transcription factors that regulate

mitochondrial genes through cooperation between the nucleus and mitochondria ⁹⁸. One essential step in mitochondrial biogenesis is activating transcription factors that regulate mitochondrial genes. These factors include peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), nuclear receptors and transcription factors. PGC-1 α is activated in response to changes in metabolic state. AMP-activated protein kinase (AMPK) is an energy sensor that detects the AMP/ATP ratio in response to mitochondrial ROS and is activated in low-energy settings. When AMPK is activated, it directly or indirectly phosphorylates PGC-1 α . The latter pathway is activated by raising the NAD-dependent deacetylase sirtuin 1 (SIRT1) ⁹⁹. PGC-1 α modulates mitochondrial biogenesis by co-activating nuclear receptors and transcription factors. Once activated, the downstream genes involved in mitochondrial biogenesis, such as protein import and assembly (TOM20, TOM70, COX17) ¹⁰⁰, respiratory components (complexes I, III, IV, and V) ¹⁰¹, and heme biosynthetic enzymes in the mitochondrial matrix ¹⁰². There is also mounting evidence that target genes are not only responsible for regulating the expression of mitochondrial translational components such as ribosomal proteins and tRNA synthetases but also for mitochondrial DNA (mtDNA) transcription and replication ^{103,104}. Furthermore, several proteins, such as mitofusin 1/2 (MFN1/2) and dynamin-related protein (DRP1), are synthesized to maintain mitochondrial dynamics ¹⁰⁵. PPAR α , a PGC-1 α transcription factor, activates the expression of copper-zinc superoxide dismutase 1 (SOD1) protein ¹⁰⁶. Thus, the number and functionality of mitochondria are ensured to meet the cellular energy demand (**Fig.1.1**).

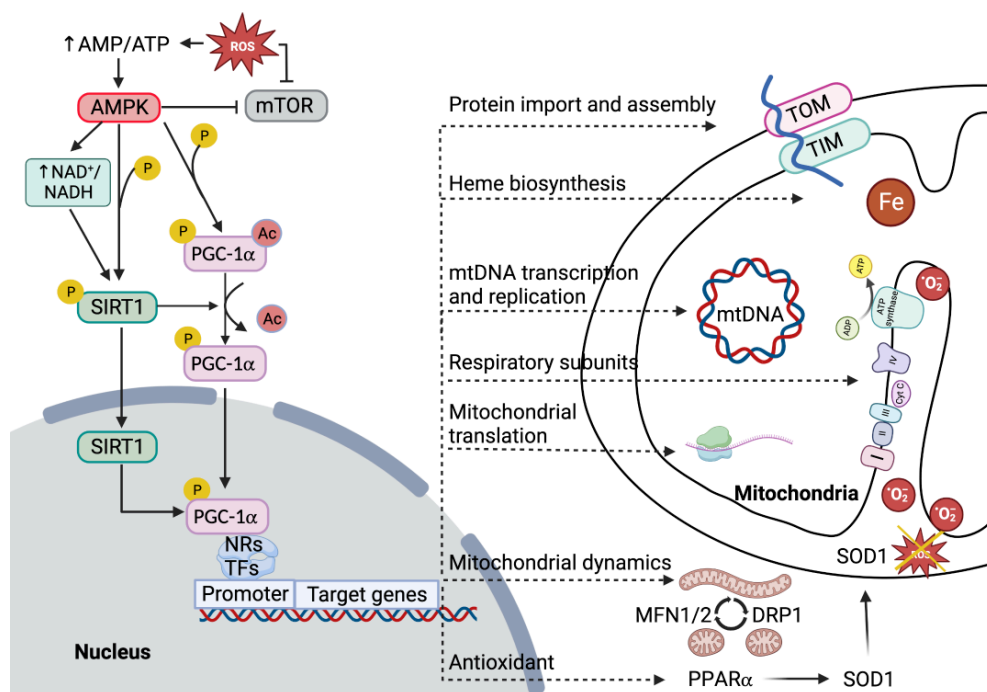


Fig.1.1 Mitochondrial biogenesis is a complex process involving several signaling pathways.

AMPK is an energy sensor that detects the AMP/ATP ratio in response to mitochondrial ROS and is activated in low-energy settings. When AMPK is activated, it raises the NAD^+/NADH ratio and phosphorylates sirtuin 1 (SIRT1), which deacetylates PGC-1 α . It can also directly phosphorylate PGC-1 α while inhibiting mammalian target of rapamycin (mTOR) action. The activated PGC1 α co-activates NRs/TFs and regulates the nuclear target genes encoding proteins involved in mitochondrial biogenesis, such as protein import and assembly (TOM20, TOM70, COX17), respiratory components (complexes I, III, IV, and V), and heme biosynthetic enzymes in the mitochondrial matrix. Furthermore, several proteins, such as mitofusin 1/2 (MFN1/2) and dynamin-related protein (DRP1), are synthesized to maintain mitochondrial dynamics. PPAR α activates the expression of copper-zinc superoxide dismutase 1 (SOD1) protein. Agreement number: QB25L8BS3A. Created with BioRender.com, accessed on 10 July 2023.

1.2.2. Mitochondrial dysfunction in aging

Aging is a chronic functional decline with multiple biological disarrangements. Mitochondrial dysfunction is one of the hallmarks of aging²⁴. It is related to many age-associated diseases, such as Cockayne syndrome¹⁰⁷, Ataxia telangiectasia¹⁰⁸, Xeroderma pigmentosum¹⁰⁹ and Werner syndrome¹¹⁰, Parkinson's disease (PD)¹¹¹, Alzheimer's disease (AD)¹¹², Amyotrophic lateral sclerosis¹¹³. Several factors are demonstrated to trigger the decline of mitochondrial function during aging, including elevated ROS production, mitochondrial DNA (mtDNA) mutations, protein oxidations, decline of mitochondrial respiratory capacity, abnormal AMPK-mediated energy metabolism, and reduced mitochondrial biogenesis^{41,114} (**Fig.1.2**).

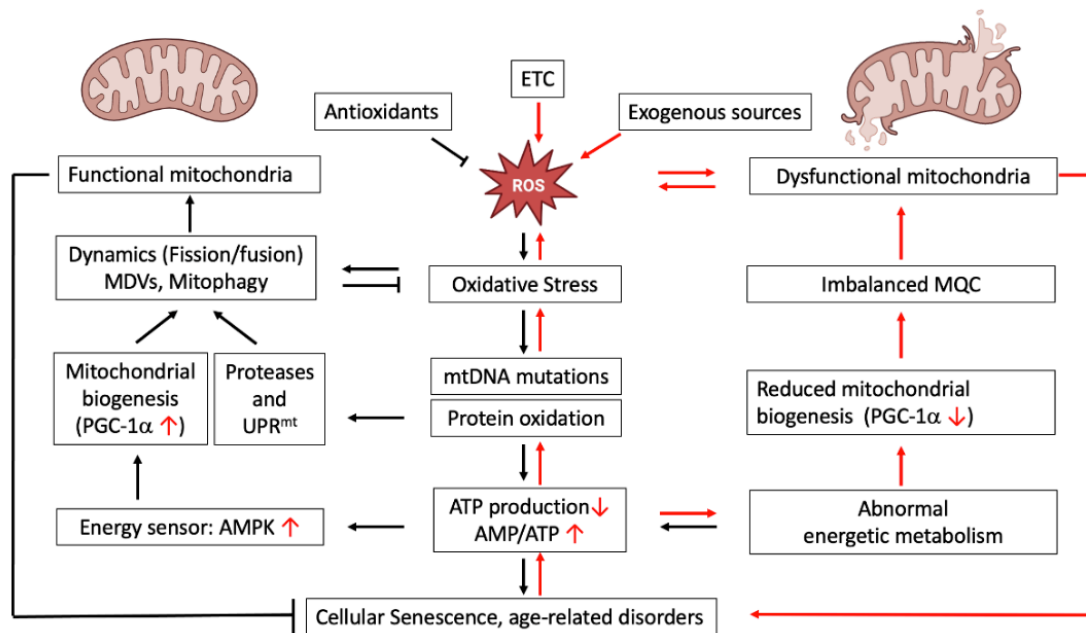


Fig1.2 Oxidative stress-induced mitochondrial dysfunction accelerates the aging process

ROS are generated from mitochondrial ETC and exogenous sources. Enzymatic and non-enzymatic antioxidants can convert ROS into less harmful by-products in physiological conditions. Excessive ROS induce oxidative stress, which leads to mitochondrial DNA mutations and protein oxidation. The abnormal products in mitochondria reduce ATP production and increase AMP/ATP ratio. AMPK, an energy sensor, could upregulate mitochondrial biogenesis and promote quality

control. However, persistently decreased production of ATP induces abnormal energetic metabolism, which causes reduced mitochondrial biogenesis, imbalanced MQC, and mitochondrial dysfunction. These aberrant changes ultimately lead to cellular senescence and age-related disorders. Agreement number: OV25L8C04J. Created with BioRender.com, accessed on 10 July 2023.

1.2.2.1 Elevated ROS production and oxidative stress in aging

As organisms age, there is a gradual decline in physiological functions, which is accompanied by increased production of ROS and oxidative stress ¹¹⁵. ROS are highly reactive molecules that are generated as a by-product of normal metabolic processes, such as cellular respiration, and can damage cellular components such as proteins, lipids, and nucleotides ⁹³. ROS production is triggered by various exogenous factors, including oxidants in foods and nutrients and endogenous oxidative reactions such as lipid peroxidation chain reactions and mitochondrial oxidative phosphorylation ¹¹⁶. ROS include superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and alkoxyl and peroxy radicals ¹¹⁷. The production of ROS is a natural consequence of various cellular processes, and the body has developed several mechanisms to counteract their damaging effects.

As we age, our body becomes less efficient in neutralizing ROS and accumulates oxidative damage¹¹⁸. The constant production of ROS may modify enzyme activities and change biomolecule structures ¹¹⁹. Enzymatic antioxidants, such as SOD1, glutathione peroxidase, glutathione-S-transferase, and catalase, convert ROS into less harmful by-products in physiological conditions ¹²⁰. Imbalanced generation and neutralization of ROS accelerate oxidative stress; vice versa, oxidative stress can also induce ROS generation ¹²¹. Oxidative stress

has been linked to various age-related diseases, including neurodegenerative disorders, cardiovascular diseases, and cancers ¹²². It is thought that oxidative stress contributes to the pathogenesis of these diseases by promoting inflammation, cellular senescence, and DNA damage¹²³.

Mitochondria are the primary site for ROS production in the cell; as such, mitochondrial function changes can significantly impact oxidative stress ¹²⁴. With age, there is a decline in mitochondrial function, leading to increased ROS production. Respiratory chain complexes I and III participate in the ubiquinone cycle in stimulating univalent oxygen reduction; complex II is responsible for superoxide anion production ⁹⁵. In the aging process, excessive ROS continually accumulate in mitochondria, causing decreased ATP production and disrupted respiratory chain activity, ultimately leading to mitochondrial dysfunction and damage ¹²⁵. In neurons, for example, high levels of mitochondrial ROS can induce neuroinflammation and neuronal death ¹¹⁸.

1.2.2.2 Higher level of mitochondrial DNA mutations in aging

While aging is a natural process that occurs in all living organisms, a combination of genetic, lifestyle, and environmental factors might cause alterations in aging process. This premature aging can significantly impact an individual's quality of life and lifespan ¹²⁶. One contributing factor is genetic mutations that affect mitochondrial function. Specifically, mutations in mitochondrial DNA can impair energy production and increase the production of ROS, further exacerbating the aging process ¹²⁷. Mitochondrial DNA consists of small circular chromosomes in mitochondria. It contains 37 genes that encode 13 proteins, 22 tRNAs, and 2 rRNAs ¹²⁸. mtDNA is hereditary and constantly affected by both exogenous and endogenous stresses. Besides, mtDNA is more vulnerable to mutations (approximately 10-fold higher) than nuclear DNA. Base excision repair,

direct reversal, mismatch repair, and possibly double-strand break repair are proven to protect mtDNA.

However, the genomes are very close to the respiratory complexes in the IMM, where ROS are generated in large quantities during oxidative phosphorylation ¹²⁹. Although the repair systems exist in mitochondria, they are still relatively limited to counteract the mutations without the protection of histone ¹³⁰. Consequently, mtDNA mutations can cause imbalanced mitochondrial respiration, accelerating ROS accumulation. This vicious circle contributes to cellular senescence and age-related disorders ¹³¹. A study shows that transgenic mice with mtDNA mutations display early aging phenotypes, such as hunchback, alopecia, grey hair, hearing loss, and weight loss, with shortened lifespans ¹³². Aging humans also exhibit higher levels of mutations in the mitochondrial genome than young individuals in their brains, hearts, and skeletal muscles ¹³³⁻¹³⁵. Further research may provide some mechanistic understanding of aging and age-related neurological disorders ¹³⁶.

1.2.2.3 Accumulation of oxidized proteins in aging

Proteins are a primary target of oxidative modifications during aging. Since mitochondria are a primary site for ROS production, mitochondrial proteins are particularly susceptible to oxidative modifications ¹³⁷. Thirteen mitochondrial gene-encoded proteins are synthesized in the mitochondrial matrix. In comparison, more than 1200 nuclear-encoded mitochondrial proteins are synthesized in the cytosol and delivered to the outer mitochondrial membrane, inner mitochondrial membrane, and inner mitochondrial space (IMS), and mitochondrial matrix. The oxidative stress causes a plethora of posttranslational protein modifications by targeting sulfur-containing moieties, such as the thiol group of cysteine and the thioether group of methionine. Cysteine residues in proteins play a crucial role in stabilizing their structures. Still, they are particularly susceptible to oxidative modification due to the vulnerability of their sulfhydryl group to attack by redox-active

substances¹³⁸. When a thiolate reacts with H₂O₂, the first by-product formed is sulfenic acid, which can undergo various reactions depending on the conditions. Sulfenic acid can form intermolecular or intramolecular disulfide bonds, which can be reversed by glutathione. However, sulfenic acid can also be further oxidized to sulfinic and sulfonic acids. Among these, sulfonic acid is the most highly oxidized thiol species, and its formation is irreversible¹³⁹. MDVs and mitophagy could degrade the oxidatively modified proteins¹⁴⁰. However, excessively oxidized proteins in mitochondria deteriorate the capability of MQC¹⁴¹. The misfolded aggregates of proteins, such as α -synuclein, amyloid-beta (A β), Tau, 43kda TAR DNA binding protein, or SOD1, have been reported to accumulate in the space of mitochondria that leads to mitochondrial dysfunction and neural degeneration¹⁴²⁻¹⁴⁵.

1.2.2.4 Decline of mitochondrial respiratory capacity and ATP production in aging

Mitochondrial health is essential for physiologic function, especially in tissues with a high ATP turnover rate. However, mitochondrial respiratory capacity and coupling quality control decline with age¹⁴⁶. The respiratory chain complexes (complexes I, II, III, and IV) and ATP synthase-complex V exist in the IMM and participate in oxidative phosphorylation. Electrons are transferred through the respiratory complexes to form ATP. In case of a sudden increase in energy demand, respiratory reserve capacity can be used to produce extra ATP through oxidative phosphorylation¹⁴⁷. However, if the respiratory reserve capacities of mitochondria are exhausted, the affected cells undergo senescence or die¹⁴⁸. Studies have shown that pharmacological inhibition or genetic manipulation of mitochondrial respiratory complexes can induce cellular senescence¹⁴⁹. Rotenone, a complex I inhibitor, upregulates senescence-associated β -galactosidase activity and ROS levels leading to cellular senescence¹⁴⁸. Deferoxamine mesylate is an FDA-approved iron chelator. It inhibits complex II activity and could either promote or prevent cellular senescence depending on

its concentration and mode of application ^{150,151}. Silencing of Rieske iron-sulfur polypeptide, a critical protein subunit of complex III, significantly inhibits ATP synthesis and leads to cellular senescence ¹⁵². Reduced levels of mitochondrial Coenzyme Q and decreased ETC efficiency are associated with age-related declines in mitochondrial function ¹⁵³.

1.2.2.5 Abnormal AMPK-mediated energy metabolism in aging

There is growing evidence suggesting a decline in energy metabolism during aging, and this process is closely connected to AMPK's positive activation in response to oxidative stress and decreased ATP production. ¹⁵⁴. AMPK triggers catabolism, such as glycolysis and fatty acid oxidation, while inhibits anabolism, such as protein synthesis ¹⁵⁵. AMPK has also been proven to be a critical modulator of aging by inhibiting the mammalian target of rapamycin (mTOR) and phosphorylation of sirtuins ¹⁵⁶. mTOR is a ubiquitously expressed serine/threonine-protein kinase within the PI3K family that controls cellular energy levels and nutritional and growth factor status ¹⁵⁷. It has been demonstrated that deleting the mTORC1 component raptor in mouse skeletal muscle induces mitochondrial dysfunction ¹⁵⁸. SIRT1 deacetylates target substrates in an NAD⁺-dependent manner. Its activity directly correlates metabolic perturbations with transcriptional outputs. Moreover, SIRT1 substrates may extend beyond transcriptional regulators to directly deacetylate the acetyl-CoA synthetase and mitochondrial biogenesis-related proteins ^{159,160}. AMPK signalling becomes less responsive as people age ¹³². When AMPK activation is insensitive to cellular stress, such as obesity or insulin resistance, energy metabolism is abnormal. Therefore, the abnormal energy metabolism caused by the insensitivity of AMPK activation can significantly affect cellular health and longevity, including imbalanced mitochondrial dynamics, reduced mitochondrial biogenesis, and, ultimately, mitochondrial dysfunction and cellular senescence ¹⁶¹.

1.2.2.6 Reduced mitochondrial biogenesis in aging

Over the past decades, studies have shown that mitochondrial biogenesis decreases with age. PGC-1 α is the master regulator of mitochondrial biogenesis. Ample evidence supports the role of PGC-1 α in aging. Increased expression of PGC-1 α could compensate for the mitochondrial dysfunction and improve aging phenotypes in mtDNA mutation mice ¹⁶². However, there is an inadequate response of PGC-1 α to exercise training in aged mice ¹⁶³. Mitochondrial transcription factor A (TFAM) is a downstream transcription coactivator of PGC-1 α . It regulates mtDNA transcription and packages mtDNA into nucleoids ¹⁶⁴. Loss of TFAM leads to less mtDNA and impairment of the electron transport chain ¹⁶⁵. However, oncogene-induced senescence shows elevated levels of both PGC-1 α and TFAM. Thus, over-activated mitochondrial biogenesis might also be detrimental to cells ¹⁶⁶.

1.2.3 Imbalanced mitochondrial quality control during aging

A series of MQC maintains mitochondrial function and homeostasis. As MQC operates through a complex network of pathways, derangements at any level may influence the entire system ¹⁶⁷. Accumulated dysfunctional mitochondria due to failure of MQC and the expansion of mitochondrial defects eventually lead to cell senescence and death ¹⁶⁸.

1.2.3.1 Mitochondrial proteolysis and aging

Mitochondrial proteolysis is the frontline of mitochondrial quality control ¹⁶⁹. Many proteases, such as the Lon protease, ATP-dependent Clp protease proteolytic subunit (ClpP), and membrane-bound proteases, maintain proteostasis in mitochondria, degrade misfolded proteins and import newly synthesized proteins. The ubiquitin-proteasome system directs damaged or misfolded proteins in the mitochondria for proteasomal degradation. The Lon and ClpP proteases in the mitochondrial matrix are responsible for the proteolysis of damaged proteins ¹. Age-dependent decline in mitochondrial proteolysis leads to the accumulation of misfolded proteins, which

promotes cellular stress and may contribute to aging. A study on mouse skeletal muscle shows that the activity of Lon levels declines with age ¹⁷⁰. Increasing chronic oxidative stress may exacerbate Lon's deleterious effects with age ¹⁷¹. Besides, there is evidence that ClpP^{-/-} mice exhibit severe growth retardation, decreased spontaneous activity, and reduced survival phenotypes as they age ¹⁷². Neurons accumulating tau proteins resulting from dysfunctional ubiquitin-proteasome systems show aberrant synaptic activities in AD patients ¹⁷³. As a result, impairment of this system could induce the accumulation of misfolded or damaged proteins, which may lead to aberrant cellular function and contribute to aging progression.

1.2.3.2 Mitochondrial dynamics and aging

Mitochondria are highly dynamic through fission and fusion. Fission breaks one mitochondrion into two, while fusion joins two mitochondria together. Fission and fusion dynamics help maintain mitochondrial number, morphology, functions, and subcellular distribution, essential to preserving mitochondrial health. GTPases of hydrolase enzymes control the mitochondrial network. The dynamic-related protein (DRP1) directs fission in mitochondria, while MFN1 and MFN2 on the outer membrane and optic atrophy 1 (OPA1) on the inner membrane mediate mitochondrial fusion. A deficiency of fission or excessive fusion often leads to a highly connected and extended mitochondrial network ^{174,175}, that may promote mitochondrial biogenesis but accumulate toxic by-products ¹⁷⁶⁻¹⁷⁸. On the other hand, insufficient fusion or additional fission may lead to mitochondrial autophagy or fragmentation ^{131,179,180}. Various senescent cells show enlarged cell bodies with complicatedly interconnected and elongated mitochondria ¹⁸¹. Low expression of p-DRP1 and high expression of MFN2 in senescent mesenchymal stem cells has been found to promote mitochondrial fusion during aging ¹⁸². However, an opposite finding shows higher expression of DRP1 but lower expression of OPA1 in the brains of aged mice ¹⁸³ (**Fig.1.3**).

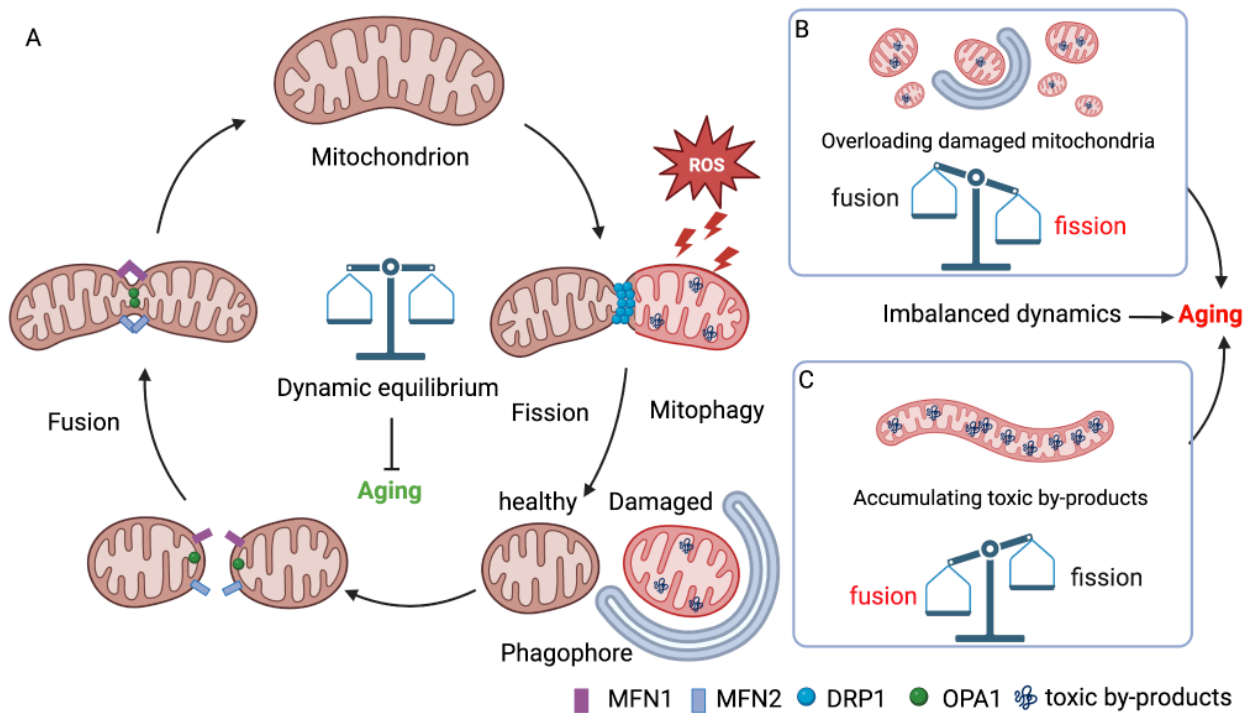


Fig.1.3 Imbalanced mitochondrial dynamics accelerates aging

(A) Mitochondrial dynamic equilibrium is essential for mitochondrial homeostasis as they regulate the mitochondrial number, shape, function, and subcellular distribution. Many GTPases are involved in the regulation of mitochondrial dynamics. The DRP1 protein directs fission in mitochondria, and MFN1 and MFN2 on the outer membrane and OPA1 on the inner membrane mediate fusion. The damaged part of mitochondria is separated by DRP1 from the healthy part and is engulfed by a phagophore to undergo mitophagy. In addition, the healthy part of the mitochondria fuses with other mitochondria to fulfil its function for anti-aging. (B) Insufficient fusion or additional fission may lead to mitochondrial autophagy or fragmentation. (C) A deficiency of fission or excessive fusion often leads to a highly connected and extended mitochondrial network that may promote mitochondrial biogenesis but accumulate toxic by-products. Imbalanced dynamics may accelerate aging. Agreement number: PO25L87F4Z. Created with BioRender.com, accessed on 10 July 2023.

1.2.3.3 Mitochondrial unfolded protein response (UPR^{mt}) and aging

UPR^{mt} is a molecular protective mechanism that helps refold misfolded and unfolded proteins in mitochondria to create a stable mitochondrial environment. UPR^{mt} retrogrades the stress signals from mitochondria to the nucleus (**Fig. 1.4**). In mammals, the accumulation of misfolded proteins in the intermembrane space and mitochondrial matrix can activate UPR^{mt} through several axes, ATF5-UPR^{mt}, HSPs-UPR^{mt}, sirtuins-UPR^{mt}, and ER α -UPR^{mt} in mammals. Studies have demonstrated that the accumulation of misfolded proteins, such as APP/A β , tau, α -Synuclein, and SOD1^{G93A}, persists in activating UPR^{mt} in age-related neurodegenerative diseases¹⁸⁴⁻¹⁸⁶. However, UPR^{mt} may not repair severely damaged mitochondria with excessive misfolded proteins and aggregates, although it is highly activated¹⁸⁷. Intriguingly, histone-3 is abnormally trimethylated in senescent cells. This condition inhibits ATF-5 from binding with DNA, resulting in the shutdown of UPR^{mt}¹⁸⁸. Consequently, the activation of UPR^{mt} has the potential to offer protective effects during the early stages of aging. However, when UPR^{mt} is excessively activated or deactivated, it can hasten the progression of aging.¹⁸⁹⁻¹⁹¹

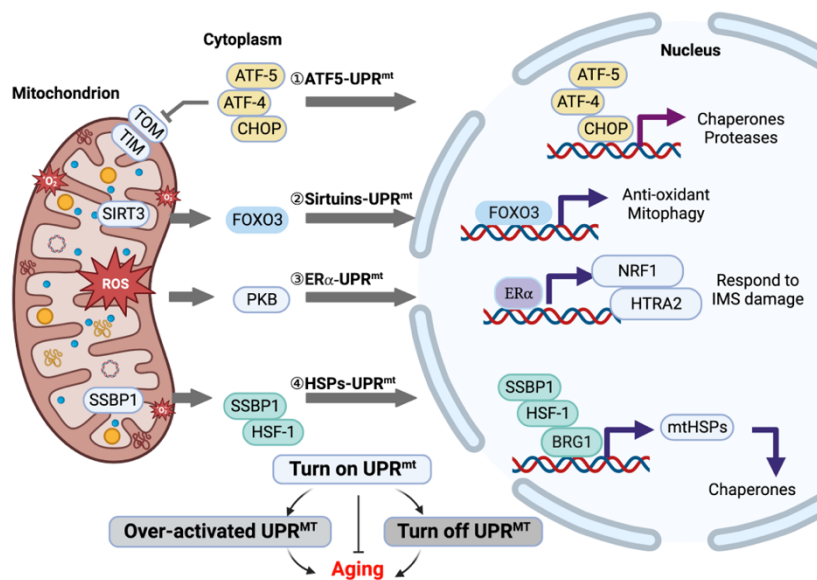


Fig. 1.4 Overactivated or inhibited mitochondrial UPR^{mt} accelerates aging

Oxidative stress activates four different axes of the UPR^{mt} in mammals. ① ATF5-UPR^{mt} axis, ② Sirtuin-UPR^{mt} axis, ③ ERα-UPR^{mt} axis, ④ HSPs-UPR^{mt}. Activation of the UPR^{mt} facilitates the degradation of misfolded proteins and contributes to the deceleration of aging. Conversely, when UPR^{mt} is excessively activated or inhibited, it leads to an acceleration of the aging process. Agreement number: GX25L8BSVR. Created with BioRender.com, accessed on 10 July 2023.

1.2.3.4 Mitophagy and aging

In recent years, many studies have focused on the role of mitophagy due to its close relationship with aging^{192,193}. Mitophagy is a mitochondrial response to various stresses, including oxidative stress, nutrient starvation, or programmed removal of mitochondria⁹² (**Fig. 1.5**). Aging impairs mitochondrial function and mitophagy and increases IL-6 levels in the cerebral vasculature, which may compromise the integrity of the blood-brain barrier and diminish cerebrovascular health with aging¹⁹⁴. Here we aim to summarize the underlying mechanisms between mitophagy and aging.

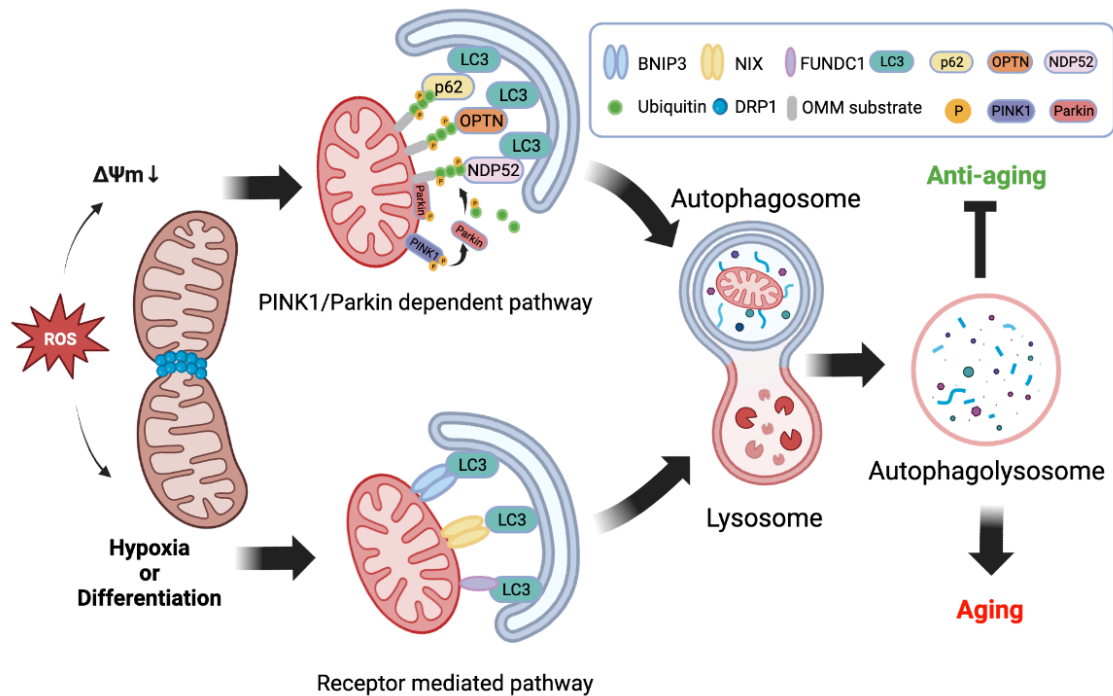


Fig. 1.5 Overactivated or insufficient mitophagy accelerates aging

In healthy mitochondria, PINK1 can be eliminated constitutively after being imported to the inner membrane by the proteasome. When a mitochondrion undergoes oxidative stress, PINK1 import is impeded, and PINK1 accumulates on the outer membrane. Once docked on the outer membrane, PINK1 recruits and activates Parkin via phosphorylating S65 in Ubiquitin and Parkin's ubiquitin-like domain. Parkin then ubiquitinates mitochondrial substrates on the OMM, allowing LC3 attached to phagophores to be recruited via autophagy adaptors such as p62, OPTN, and NDP52. The phagophore swells and engulfs the mitochondrion, forming an autophagosome that merges with a lysosome to be degraded. Receptor-mediated mitophagy: The mitophagy receptor NIX, FUNDC1, and BNIP3 are strongly activated during reticulocyte maturation. This causes an increase in receptor levels on the OMM, where they can bind to LC3 conjugated to a phagophore through its LIR. After being engulfed by an autophagosome and fused with a lysosome, the

mitochondrion is destroyed by lysosomal hydrolases. However, mitophagy induced by consistent accumulation of misfolded proteins and aggregates might be over-activated or even turned off in advancing age. Besides, insufficient mitophagy has also been found to induce mitochondrial dysfunction and cellular senescence. Agreement number: ZG25L8BXAF. Created with BioRender.com, accessed on 10 July 2023.

1.2.3.4.1 PINK1/Parkin-dependent mitophagy in aging

The phosphatase and tensin homolog-induced putative protein kinase 1 (PINK1) and E3 ubiquitin ligase Parkin are significant factors in the PINK1-Parkin pathway. In physiological conditions, PINK1 is imported into the mitochondrial matrix through translocase of the outer/inner mitochondrial membrane (TOM/TIM) complex and degraded by mitochondrial proteases. Under stressful situations, PINK1 is blocked on the depolarized OMM to recruit cytosolic ubiquitin and Parkin. PINK1 phosphorylates Parkin, causing Parkin's E3 ligase function to be activated. The activated Parkin is then recruited to the depolarized mitochondria and ubiquitinates outer mitochondrial substrates ¹⁹⁵. Although it's still unclear how these autophagy components are recruited to separated membranes forming around mitochondria, adaptor proteins, including p62, Optineurin, and nuclear dot protein, bind to both ubiquitin chains and microtubule-associated protein 1A/1B-light chain 3 (LC3) /GABARAP family members. After that, the autophagosome is transported to the lysosome for digestion ¹⁹⁶. It has been demonstrated that PINK1-mediated mitophagy has a protective role against mitochondrial dysfunction ¹⁹⁷. Mutations in both PINK1 and Parkin have been discovered in PD ^{198,199}. Evidence shows that mitophagy is also impaired in the hippocampus of AD patients and a mouse model of AD ^{200,201}. Mitophagy reduces insoluble A β and protects against cognitive impairment in an AD mouse model via microglial phagocytosis

of extracellular A β plaques and inhibition of neuroinflammation ²⁰². Hence, mitophagy presents itself as a potential therapeutic intervention. Nevertheless, recent studies indicate that mitophagy might contribute to the acceleration of aging. This occurs through the activation of the PINK1/Parkin pathway-induced mitophagy by advanced glycation end products, potentially leading to the excessive degradation of mitochondria ^{203,204}. These researchers imply a potential strategy to impede the senescent state by inhibiting the activity of mitophagy. In my view, the discrepancy may result from the extent of mitophagy under different situations. Mitophagy helps the elimination of damaged mitochondria, but excessive mitophagy might impair mitochondrial function, which in turn accelerates aging ²⁰⁵.

1.2.3.4.2 Receptor-mediated mitophagy

Receptor-mediated mitophagy can selectively remove damaged mitochondria through mitochondrial receptors. Both B-cell lymphoma-2 (BCL2) /adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and BNIP3L (NIX) belong to the BH3-only domain proteins of the BCL2 family. BNIP3 exists as a monomer in the cytosol but becomes a homodimer and recruits to OMM under hypoxia or differentiation stimulation ²⁰⁶. Then the receptors interact with LC3 proteins localized in autophagosomes, which induce mitophagy ²⁰⁷. Another mitophagy receptor, NIX, also known as BNIP3L, has 53-56% amino acid sequence identity with BNIP3. It is dimerized under stress conditions, enabling mitochondria's engulfment by autophagosomes to initiate mitophagy ²⁰⁸. Other mitophagy receptors on OMM include the FUN14 domain containing 1 (FUNDC1) ²⁰⁹. In physiologic conditions, FUNDC1 is phosphorylated by the protein SRC kinase and casein kinase 2, reducing interaction with LC3 ²¹⁰. Under stressful conditions, mitochondrial phosphoglycerate mutase 5 could dephosphorylate FUNDC1 to activate mitophagy ²¹¹. NIX can induce mitophagy in compensating for mutations of PINK and Parkin ²¹². BNIP3/NIX and

FUNDC1 mitophagy may compensate for defective PINK1/Parkin-mediated mitophagy ²¹³. But lower mRNA levels of Nix, bnip3, fundc1, Pink1, Parkin, autophagy-related proteins 5 (Atg5), Atg12, and Atg13, lead to insufficient mitophagy and may lead to mitochondrial dysfunction and cellular senescence ²¹⁴.

1.2.3.5 Mitochondrial-derived vesicles (MDVs) and aging

MDVs are mitochondrial membranous protrusions that act as novel mitochondrial quality control and may also play a vital role in health, aging, and disease ²¹⁵. Although MDVs are considered the piecemeal of mitophagy, they are distinct mitochondrial processes. Firstly, MDVs are generated from healthy mitochondria, whereas mitophagy occurs in depolarized ones ²¹⁶. Secondly, MDVs with single or double membranes have only 70-150nm in diameter, which are much smaller than mitophagosomes (>300 nm in diameter) ²¹⁷. Thirdly, MDVs are independent of mitophagy-associated proteins, DRP1, ATG5, LC3, Beclin-1, or RAB9 ^{192,218,219}. As a result, MDVs could compensate for the loss of mitophagy to maintain mitochondrial homeostasis ²²⁰. Seventy-two proteins are discovered in MDVs, including 31% oxidative phosphorylation proteins and TIM chaperones, indicating MDV transportation might be chaperone-mediated ²²¹. TOM20 and mitochondria-anchored protein ligase (MAPL) are mitochondrial outer-membrane proteins. Pyruvate dehydrogenase (PDH) is a protein in the mitochondrial matrix. Although the fate of MDVs is still unknown, MDVs are selected by their intended target and the type of mitochondrial stress ^{222,223}. MAPL⁺ MDVs are targeted at peroxisomes for degradation ²²³. TOM20⁺ MDVs are associated with DRP1 and mitochondrial Rho GTPase 1/2 and will then be degraded within the lysosomes ^{218,221,224,225}. On the other hand, a part of PDH⁺ MDVs shares the same PINK1/Parkin trafficking pathway with mitophagy ²²⁶. They are delivered by a ternary SNARE complex, syntaxin-17, SNARE-binding protein synaptosome-associated protein of 29 kDa (SNAP29), and

vesicle-associated membrane protein 7 (VAMP7) to lysosomes for degradation ²²⁷. In addition, Sorting nexin 9 (SNX9) is responsible for protein sorting and transport, vesicle formation, and endocytosis. SNX9 accumulates on the mitochondrial surface during mitochondrial antigen presentation to modulate the production of PDH⁺ MDVs ²²⁸. OPA1 and SNX9-directed PDH⁺MDVs package damage-associated molecular patterns into multivesicular bodies (MVBs) ^{224,229}. Then MVBs fuse with cellular membranes and release MDVs to the extracellular space, stimulating mitochondria-driven immune responses (**Fig.1.6**). Several pieces of evidence on the extracellular vesicles (EVs) are discovered to transport to the adjacent cells and proven to trigger innate immunity, which might accelerate cellular senescence ^{90,230,231}. In older adults with physical frailty and sarcopenia, elevated EV levels containing mitochondrial components have provided additional evidence ²³². Studies also indicate EVs are more abundant in the serum samples of PD patients than in the age-matched control group without PD, indicating a mechanism for cells to dispose of damaged mitochondria ⁸¹. Besides, mitochondria couldn't secrete MDVs sufficiently in older PD patients, which may explain dysfunctional mitochondria lose their ability to degrade the misfolded proteins through MDVs in PD ²³³. Hence, MDVs are like a two-edged sword, either delaying or accelerating aging. Understanding the role of MDVs in mitochondrial homeostasis may help discover new tools and strategies for preventing or treating age-related diseases.

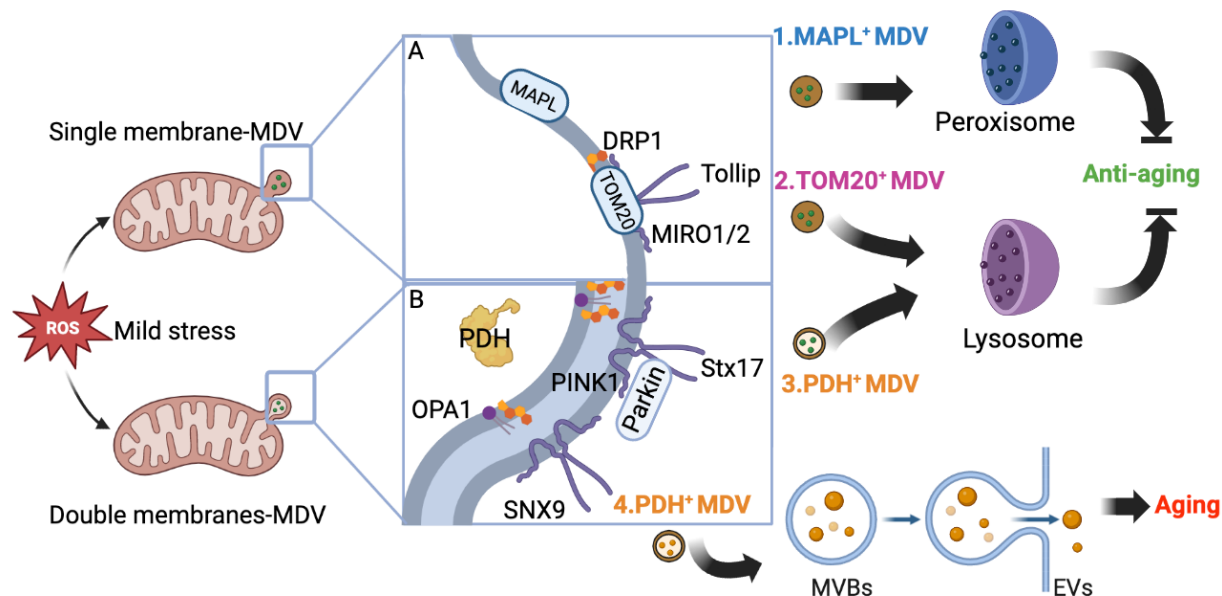


Fig. 1.6. Insufficient MDVs or released MDVs as extracellular vesicles may contribute to aging

Different molecular pathways and destinations may cause MDVs to delay or accelerate aging. MAPL⁺ MDVs with a single-layer membrane are directed to the peroxisome, while TOM20 + MDVs are initiated by MIRO1/2 and DRP1 and then transported to the lysosome by Tollip. Double-membrane PDH⁺ MDVs have two distinct destinations. PINK1/Parkin-dependent PDH⁺ MDVs are conveyed to lysosomes for degradation by a ternary SNARE complex (Syntaxin-17, SNAP29, and VAMP7). The oxidized by-products in the previously mentioned MDVs may be digested by lysosomes, which may assist mitochondria in staying healthy and delaying cellular senescence. When OPA1 and SNX9-directed PDH⁺MDVs exceed the capacity of lysosomes, they are organized into multivesicular bodies (MVBs) and released as extracellular vehicles. The complex contents may be transported to neighbouring cells, thereby hastening cellular senescence. Agreement number: MJ25L8AX3F. Created with BioRender.com, accessed on 10 July 2023.

In summary, MQC is a double-edged sword. They promote mitochondrial healthy by degrading harmful by-products and isolating damaged mitochondria. However, if MQC persists beyond a threshold, it can have negative consequences. Mitochondrial biogenesis can be hindered by proteasome competition for non-imported cytosolic precursor proteins ²³⁴. Accumulation of misfolded or damaged proteins due to the over-activation of UPR^{mt} has been linked to aberrant cellular function and aging ²³⁵. When both fission and mitophagy are overactive, cellular senescence may be accelerated ²⁰⁵. Overloaded MDVs might release toxic by-products into the extracellular space ²³⁶. Therefore, selective, efficient, and gentle activation of MQC is essential for MQC. It implies that therapeutic interventions at various stages of MQC should be modulated appropriately. Additionally, the molecular and metabolism of mitochondria should be monitored simultaneously.

1.2.4. Therapeutic interventions on aging through targeting mitochondria

1.2.4.1 Targeting mitochondrial biogenesis

Strategies aimed at mitochondrial biogenesis generally include manipulating cellular biochemistry and energy homeostasis. Regular physical activity is known to increase mitochondrial biogenesis in various tissues. Exercise increases the demand for ATP, which signals the cell to produce more mitochondria to meet this increased energy demand ²³⁷. High-intensity interval training can help prevent various metabolic diseases by increasing glucose utilization. It can increase cellular energy sensors, such as PGC-1 α , AMPK, SIRT1 and ROS in skeletal muscle ²³⁸. Studies have shown that exercise rescues age-related mitochondrial network fragmentation and restores impaired submaximal ADP-stimulated respiration ²³⁹. This occurs because exercise increases PGC-1 α expression and activity, which leads to an upregulation of mitochondrial biogenesis and function

^{240,241}.

AMPK is another crucial regulator of cellular energy homeostasis and is an upstream modulator of PGC-1 α . Due to its role as a central lipid and glucose metabolism regulator, AMPK is a potential therapeutic target for treating age-related disorders, such as type-II diabetes mellitus, obesity, neurodegenerative diseases, and cancer ²⁴². Dietary restriction (DR) is described as a decrease in food intake that does not result in malnutrition. It has been found to delay the development of age-related diseases and prolong the lifespan of numerous creatures ²⁴³. DR refers to intermittent fasting, a fraction of the entire diet consumed by ad libitum-fed animals or a limitation in a specific macro/micronutrient ²⁴⁴. Intermittent fasting has been proven to inhibit mTOR activity, decrease expression of Sirt3, and increase cellular NAD⁺/NADH which could activate AMPK/PGC-1 α ^{245,246}. Besides, a calorie restriction (CR) diet has also been demonstrated to improve mitochondrial biogenesis by activating AMPK ²⁴⁷. It should be emphasized that DR levels beyond 60% may harm the organism's longevity and general health ²⁴⁸.

Dietary supplements and potential drugs, known as calorie restriction mimetics or energy restriction mimetics, are proposed as a hypothetical class that would imitate the significant anti-aging benefits of CR. Resveratrol, a CR mimetic, increases muscle performance in old individuals by increasing mitochondria mass and elevating Nrf-1 and TFAM ^{249,250}. Metformin, the first-line anti-hyperglycemia for type 2 diabetes mellitus, is recently discovered as a blocker of complex I of the mitochondrial electron transport chain to decrease ATP levels and boost the AMP/ATP ratio, which in turn increases AMPK activation and reduce ROS production ^{251,252}. However, Lilia *et al.* found metformin aggravates aging-related mitochondrial failure, resulting in lethal ATP depletion ²⁵³. Thus, metformin therapy in later life may reduce cell survival and shorten longevity ²⁵⁴. Rhizoma Coptidis, as an anti-aging natural product, has pharmacologic potential in combating aging-related diseases by activating AMPK cellular kinase ²⁵⁵. Rapamycin, an mTOR inhibitor,

improves mitochondrial biogenesis and extends mice's lifespan²⁵⁶. Thus, AMPK and mTOR might be critical mediators behind mitochondrial effects on longevity and might provide a promising therapeutic target in treating aging-related diseases¹¹⁴.

1.2.4.2 Targeting mitochondrial dynamics

The dynamics in mitochondrial shape and networks impact mitochondrial function and quality. Studies have proved that co-inhibition of fusion and fission by intermittent fasting could reduce the lifespan of yeast²⁵⁷. In comparison, 20% of female and 12% of male flies lived longer than their typical lifespan if induced with DRP1-mediated fission in the midlife of *Drosophila melanogaster*¹⁷⁴.

A recent study also implies that exercise improves muscle function by regulating mitochondrial dynamics via the AMPK pathway²⁵⁸. Impairing mitochondrial fission or fusion negates the beneficial effects of constitutive AMPK activation on physical fitness preservation in older people²⁵⁸. Resveratrol is generally used as a dietary supplement. It suppresses mitochondrial fusion and promotes fission through the PINK1/parkin pathway²⁵⁹. However, a study shows that resveratrol inhibits both fusion and fission to modulate mitochondrial quality in the diabetic muscle of mice²⁶⁰. Therefore, strategies for modulating the balance of mitochondrial fission and fusion appear critical for anti-aging outcomes.

1.2.4.3 Targeting UPR^{mt}

Previous studies have discovered that activation of UPR^{mt} contributes to longevity⁸⁹. For instance, ROS elimination by activating the effector of UPR^{mt}-ClpP delays liver cell senescence²⁶¹. A study on Honokiol (HKL, C₁₈H₁₈O₂), a pharmacologically active natural compound isolated from the bark of *Magnolia Officinalis*, showed that HKL activates sirtuin 3 (SIRT3)-UPR^{mt} signalling and improves cognitive impairment in a mouse model of AD²⁶². UPR^{mt} is, therefore, a possible

therapeutic target. It is also reported that protein disulfide isomerase 6 (PDI-6) is expressed in the endoplasmic reticulum and interacts with EGL-20 through disulfide bonds of PDI-6. Overexpression of PDI-6 through UPR^{mt} activation is sufficient to keep Wnt/EGL-20 protein levels stable during aging in *C. elegans* ²⁶³. Still, the long-term effects of this activation remain uncertain because prolonged UPR^{mt} activation can result in mitochondrial dysfunction, and the transcription of numerous unknown functional genes may induce cancer ^{264,265}.

1.2.4.4 Targeting mitophagy

Pharmacological therapies targeting mitophagy have generated considerable interest and may have significant translational potential. A study that demonstrates a protective function for mitophagy in neurons and microglia raises the possibility that mitophagy induction is advantageous for other glial and non-neuronal cells ²⁰². Several strategies are applied to modify mitophagy signalling pathways or directly target mitophagy proteins. Other evidence indicates that increased mitophagy through activation of the PINK1/Parkin pathway is a promising therapeutic target for PD ¹⁹⁶. Metformin is a natural SIRT1-activating compound with an anti-aging property by activating PINK1/Parkin-mediated mitophagy ²⁶⁶. Rapamycin promotes mitophagy by inhibiting mTOR and elevating PINK1, Parkin, and the pro-autophagic protein BECN1 ²⁶⁷. Besides, Atgs play a vital role in autophagosome formation. Atg32 is one of the mitophagy-specific receptors anchored on the OMM. It acts with Atg11 and Atg8 at the phagophore assembly site for lysosome degradation ²⁶⁸. In addition, Atg2-Atg18/Atg9 complex could induce mitophagy to keep mitochondrial integrity in *Drosophila* ²⁶⁹. In recent years, several natural plant extractions and postbiotic metabolites have been shown to promote mitophagy. *Gentiana iridescent*, a biennial herbaceous species, extends the lifespan of Yeast by activating Atg32 to promote mitophagy ²⁷⁰. *Lepidium meyenii*, a relative of the radish, improves cognitive function in middle-aged mice by upregulating

autophagy-related proteins, LC3, Atg7, and Beclin1 ²⁷¹. As a postbiotic metabolite, Urolithin A is a polyphenolic molecule found in pomegranate, cherries, and walnuts. In pre-clinical models of aging and age-related diseases, urolithin A has been demonstrated to stimulate mitophagy and improve mitochondrial function. A recent study shows that Urolithin A enhances muscle strength, exercise performance, and indicators of mitochondrial health in a randomized clinical trial in middle-aged people ²⁷². Oleanolic acid is a pentacyclic triterpenoid found in nature that has been shown to have antioxidant and cyclooxygenase (P450) modulating effects. Treatment with oleanolic acid attenuates aging-related alterations in mitophagy via regulating LC3B, p62, TOM20, and FUNDC1, but not BNIP3 or Parkin ²⁷³. Kaempferol and Rhapontigenin, two mitophagy inducers, enhance memory in nematode and rodent models of AD by increasing the survival and functioning of glutamatergic and cholinergic neurons and decreasing A β and tau pathologies ^{201,274}. Besides, exercise is proven to boost mitochondrial fission and mitophagy through the PGC1 α /FNDC5/irisin pathway ²⁷⁵. Through FUNDC1-dependent mitophagy, exercise shields elderly mice from coronary endothelial senescence ²⁷⁴. Short- and long-term exercise training causes changes in the levels of LC3-II and the ubiquitin-binding protein p62 ²⁷⁶. However, the pathways responsible for these exercise-induced mitochondrial adaptations remain controversial as other studies have reported a decreased adaptability of old muscles to exercise training compared to young ^{277,278}.

1.2.4.5 Targeting MDV transportation

The role of MDVs in aging is still debatable. On the one hand, intracellular MDV formation, as earlier mitochondrial quality control, is cytoprotective. Evidence indicates MDVs protect cardiomyocytes against apoptosis and ameliorate hypoxia-induced damages ²⁷⁹. Ramirez et al. discovered cannabidiol, a drug for neurological disorders, could induce the production of MDVs

by activating the PINK/Parkin pathway dose-dependently and repairing mitochondrial damage through opening permeability transition pores²²⁶. On the other hand, MDVs could be released into the extracellular space²¹⁵. There is evidence supporting that whole mitochondria in extracellular vesicles improve cardiac function in post-myocardial infarction animal models²⁸⁰. Whereas the destination and influence of the MDV-rich EVs (MDV-EVs) are still obscure. To date, EV-targeting therapeutic strategies include inhibiting EV secretion and absorption and eliminating EV circulation²⁸¹. Given the critical role of MDVs in regulating mitochondrial quality control, there is a growing interest in targeting MDV-EVs as a potential therapeutic strategy for anti-aging therapy. By manipulating MDV-EVs' content, it may be possible to modulate cellular function and mitigate the effects of aging-related changes.

Due to the multifaceted characteristics of neurodegenerative diseases, more comprehensive therapeutic interventions may be required. It is necessary to combine non-pharmacological methods and lifestyle modification with multi-targeted pharmaceutical therapies¹³⁶.

1.2.5 Conclusions and Prospects

A growing body of evidence implicates MQC provides new avenues for therapeutic intervention in aging and age-related disorders⁵⁷. **Table 1** summarises the various anti-aging approaches that target mitochondria quality modulation. Although mitochondrial dysfunction has been involved in the pathophysiology of age-related disorders, imbalanced MQC has only recently been identified as a critical contributor to disease pathogenesis²⁸². Therefore, the role of MQC must be elucidated to comprehend the mechanism of aging from the perspective of various fields of mitochondrial maintenance in response to stress. For instance, mitochondrial proteases may serve as the first defence against misfolded proteins²⁸³. At the same time, UPR^{mt} aids in refolding misfolded proteins by communicating with the nucleus, in which the target genes also modulate

mitochondrial proteases, dynamics, and mitophagy²⁸⁴. Although MDVs and mitophagy share the same molecular modulators (PINK1 and Parkin), they are found in different conditions. Damaged proteins are first disposed of via MDVs, followed by partial or entire organelle recycling via mitophagy²⁸⁵. Studies discovered MDVs compensate for the loss of mitophagy to maintain mitochondrial homeostasis²²⁰. Other studies found that MDVs could be released as exosomes and transported to the adjacent cells to trigger innate immunity, which might accelerate cellular senescence^{90,230,231}. Thus, the relationship between MDVs and mitophagy in aging is still obscure. Either overactivation or loss of MQC function might hasten the aging process (**Fig.1.7**). In addition, the function of mitochondria may not decline linearly throughout the aging process. The activity of mitochondria in middle age demonstrates a compensatory increase in the disposal of damaged mitochondria, whereas mitochondrial dysfunctions develop in old age⁸⁴. As a result, maintaining functional mitochondria and metabolic regulation is essential for long-term health.

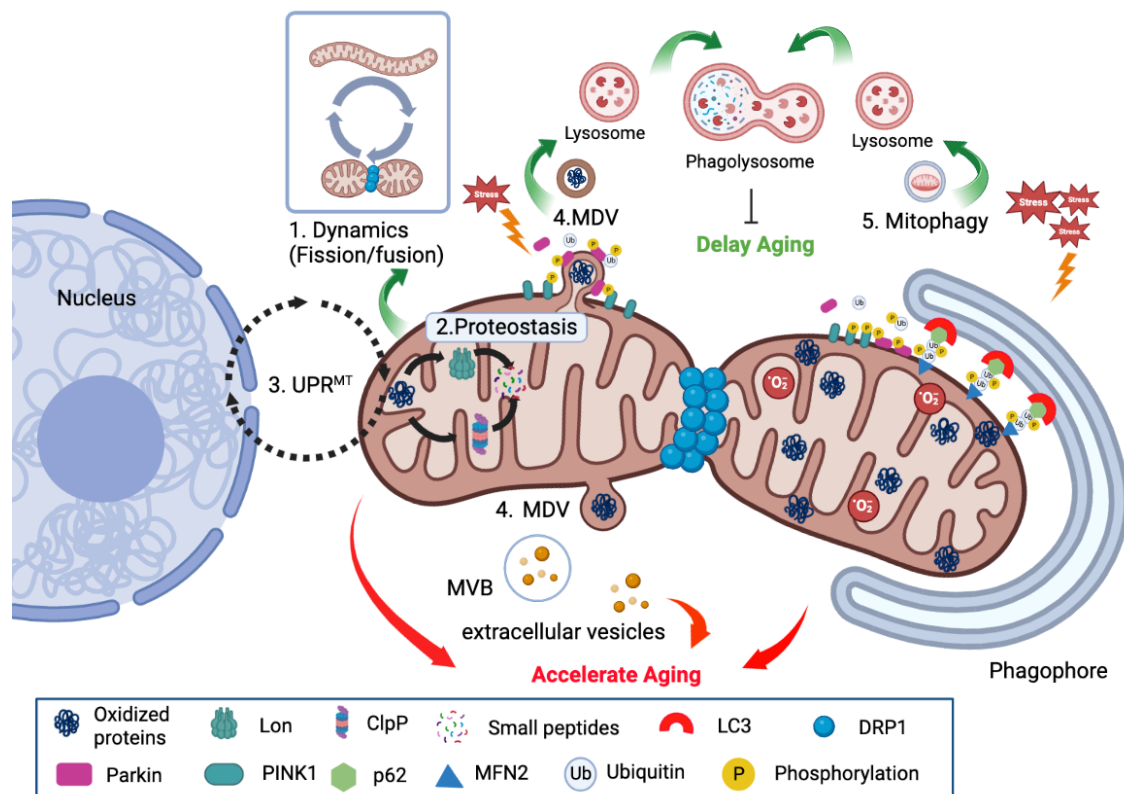


Fig. 1.7 An imbalance in mitochondrial quality control contributes to aging

Mitochondria are highly dynamic organelles that endure coordinated cycles of fission and fusion. Mitochondrial proteases and UPRmt maintain mitochondrial proteostasis through refolding the misfolded proteins or proteolysis into small peptides. Oxidative stress activates PINK1/Parkin-dependent pathway to induce MDV formation and mitophagy. On the other hand, single/double membrane MDVs could deliver the oxidized by-products to lysosomes in physical condition or under mild oxidative stress. On the other hand, severe or prolonged oxidative stress may induce mitochondrial depolarization and mitophagy. However, overactivation or inhibition of any form of MQC may accumulate oxidized proteins, accelerate aberrant energy metabolism, and induce mitochondrial dysfunction-induced senescence. Agreement number: FG25L8DDR. Created with BioRender.com, accessed on 10 July 2023.

Table1 Interventions on aging through targeting mitochondria

Target	Intervention	Mechanism of action	References
Mitochondrial biogenesis	Exercise	Stimulation of PGC-1 α and AMPK	240,286
	Intermittent fasting	Inhibition mTOR activity	245
		Decreased expression of Sirt3	246
		Increased cellular NAD ⁺ /NADH ratio	
	Calorie restriction	Activation of AMPK	247,252

	Resveratrol	Increasing mitochondrial mass	249,250
		Elevation of Nrf1 and TFAM	
	Metformin	Activation of AMPK	251,252
	Rhizoma Coptidis	Activation of AMPK	255
Mitochondr ial dynamics	Rapamycin	Inhibition of mTOR	256
	Intermittent fasting	Co-inhibition of fusion and fission	257
	Exercise	Regulation of mitochondrial fission, fusion AMPK activation	258
	Resveratrol	Promotion of fission and suppression of fusion via PINK1/Parkin pathway	259
UPR ^{mt}	Resveratrol	Inhibition of both fusion and fission	260
	Honokiol	Activation of SIRT3-UPR ^{mt}	262,287
Mitophagy	PDI-6	Activation of Wnt / EGL-20	262,263
	Metformin	Enhancing the SIRT1 /PINK1 /Parkin pathway	266
	Rapamycin	Inhibiting mTOR Elevation of PINK1, Parkin and BECN1	263,266,267

	Atgs	Activation of Atg32/Atg11/Atg9	269
	Gentiana iridescent	Activation of Atg32	267,270
	Lepidium meyenii	Upregulating LC3, Atg7, and Beclin1	270,271
	Urolithin A	Activation of PINK1/Parkin pathway	272
	Oleanolic Acid	Modulation of FUNDC1, LC3B, p62, TOM20	272,273
	Kaempferol Rhapontigen in	Activation of PINK1/Parkin pathway	201
		Modulation of FUNDC1, LC3-II, p62	273,274
	Exercise	Activation of PGC1 α /FNDC5/irisin pathway	275
		Modulation of LC3-II and p62	(Joseph et al., 2016)
MDVs	Cannabidio	Activation of PINK1/Parkin pathway	226,276,288
	Mitochondri a-rich EVs	Restoration of mitochondrial biogenesis via PGC-1 α	226,280,281

1.3 Copper-zinc superoxide dismutase (SOD1) and aging

1.3.1 SOD1 gene

The *SOD1* gene is located on chromosome 21 (region 21q22) in humans ²⁸⁹ and chromosome 16 (region 16B4 - ter) in mice ²⁹⁰. The *SOD1* gene has five exons and four introns. The TATA and CCAAT boxes and other extremely conserved GC-rich areas have consistently been in the proximal promoter region in both species ²⁹¹. Several poly-(A) signal sequences that terminate mRNA species of various lengths may be found at the 3' terminus of the *SOD1* gene. Several potential binding sites for the transcription have been identified in the promoter region of the human *SOD1* gene ²⁹². The specificity protein 1 and early growth response protein 1 transcription factors are involved in the basal and induced expression of human SOD1 ²⁹³. Over 180 unique mutations have been reported across the five exons of the SOD1 gene, resulting in more than 160 disease-associated variants within the 154-amino-acid SOD1 sequence ²⁹⁴. For example, Amyotrophic lateral sclerosis (ALS) has been linked to more than 150 distinct mutations in the *SOD1* gene ²⁹⁵. Mutations in SOD1 are believed to cause misfolding and aggregation of the protein within motor neurons and astrocytes, the cell types implicated in the development and progression of ALS ²⁹⁶.

1.3.2 SOD1 protein structure

The native form of SOD1 protein (32kDa) consists of a homodimer with two subunits, each containing one copper and one zinc atom. Zinc is crucial in preserving the stability of the SOD1 protein, whereas copper is involved in enzymatic activity ²⁹⁷. When translated, the SOD1 protein folds into an eight-strand Greek-key β -barrel. The shorter strands 4, 5, 7 and 8 are torsion-prone, containing bulges which allow the metal to be incorporated into the active site. Unlike the active site, strands 1, 2, 3 and 6, located on the opposite side of the barrel, are regular with minimal

torsion ²⁹⁸. In general conditions, most SOD1 is expressed in the cytosol. They also appear in the nucleus, lysosome, and mitochondrial IMS ²⁹⁹. It is abundant in neurons and other cells with high metabolic activity ³⁰⁰. A fraction of SOD1 monomer in cytosol transports into IMS, undergoing disulfide bond formation, zinc and copper insertion, and protein dimerization. After reaching its mature form, SOD1 remains inside IMS to fulfil its functions ²⁹⁶.

The subunit of SOD1 has four cysteine residues: Cys 6, Cys 57, Cys 111 and Cys 146. A disulfide bond between Cys57 and Cys146 can form a highly stable homodimer. Both Cys 6 and Cys 111 residues in hSOD1 possess thiol (-SH) groups that are susceptible to oxidation. However, the SH-group in Cys 6 is concealed beneath a beta strand. In contrast, Cys 111, which is unique to humans and great apes¹³⁸, is particularly prone to oxidation because of its exposed location on the surface of SOD1.

Typically, wild-type eukaryotic SOD1 is extremely stable, maintaining an intra-subunit disulfide link even in the reducing cytosolic environment and remaining active throughout a wide range of denaturing conditions. Loss of the Cu/Zn cofactors and breakage of the disulfide bonds may result in pathogenic misfolding of human SOD1, as its highly stable quaternary structure (homodimer) is regulated by its metal occupancy and its C57-C146 disulfide state ³⁰¹. It has been observed that the metalation of the molecule and the condition of the intra-subunit disulfide bonds might affect each other ³⁰². The posttranslational intra-subunit disulfide bond formation is essential for keeping SOD1 in its functional homodimer state (along with the metal ions), and its breakdown can lead to metal loss ³⁰³. This is supported by experimental findings demonstrating that severing intra-subunit disulfide bonds can untether interface loop IV, making it more flexible and hence decreasing metal binding ³⁰⁴. The proper coordination of Cu/Zn to SOD1 is essential for disulfide bond formation and structural preservation ³⁰⁵. Copper chaperone for superoxide dismutase (CCS)

was recently discovered to be induced by Cu coordination. In contrast, Zn coordination stimulates the local folding of two disordered loops into a catalytic subdomain and stabilizes the structure of SOD1³⁰⁶.

1.3.3 SOD1 Functions

1.3.3.1 Regulation of antioxidant response

SOD1 is a protein that functions as an antioxidant enzyme in cells, protecting them from oxidative stress. Its primary function is to catalyze the conversion of superoxide radicals, a type of reactive oxygen species, into less harmful substances. More specifically, SOD1 converts harmful superoxide radicals to oxygen and less toxic hydrogen peroxide. Glutathione peroxidases, peroxiredoxins, or catalase further convert hydrogen peroxide into water and oxygen³⁰⁷. Loss of SOD1 activity leads to the generation and accumulation of oxidative damage, such as protein carbonylation, protein oxidation, lipid peroxidation, and DNA breakage³⁰⁸.

1.3.3.2 Regulation of genomic stability

Besides being an antioxidant, SOD1 could also enter the nucleus and prevent genomic damage³⁰⁷. H₂O₂ is the primary ROS signal that governs the nuclear translocation of SOD1 to prevent oxidative genomic damage³⁰⁹. The nuclear localization of SOD1, lacking a conventional nuclear localization sequence, is likely facilitated through an unconventional nuclear localization sequence or by its interaction with another nuclear protein. The transportation of SOD1 protein to the nucleus is facilitated by import receptors that specifically recognize short amino acid sequences referred to as nuclear localization sequences. Export receptors are responsible for protein export by binding to nuclear export sequences of cargo proteins³¹⁰. When entering into the nucleus, SOD1 functions as a nuclear transcription factor, contributing to the activation of genes associated with oxidative stress resistance. These genes encompass various categories, including those involved in DNA

damage repair and the maintenance of genomic stability (e.g., *Mec1*). SOD1 also influences the expression of genes associated with oxidative stress resistance, such as the cytoplasmic and mitochondrial thioredoxin peroxidases *Tsa2* and *Prx1*. Additionally, SOD1 plays a role in regulating genes involved in DNA replication stress, such as the large subunit of ribonucleotide-diphosphate reductase (*Rnr3*). Moreover, SOD1 affects genes associated with iron and copper homeostasis, including the iron/copper reductases *Fre1/3/8* ^{309,311}. The DNA-binding motif most commonly associated with SOD1 is believed to be the sequence 5'-GACGACGAA-3'³⁰⁷. SOD1 binds with DNA through direct contact with the base sequence to form a three-dimensional structure of the SOD1: DNA complex ³¹². However, cells expressing heterodimers with mutant *hSOD1* (*A4V*, *L38V*, *G93A*, and *G93C*) and *hSOD1*^{WT} in cytoplasm impair the translocation of *hSOD1* into the nucleus, in turn, reduce the efficiency of the antioxidant response and shorten the lifespan of cells ³¹³. Li *et al.* found ALS-associated mutations of SOD1 reduced *SOD1*^{WT} nuclear localization and interfered with the transportation of DNA repair-processing proteins, such as p53, APEX1, HDAC1 and FUS. The accumulated DNA damage leads to the malfunction of motor neurons and causes neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) ³¹⁴.

1.3.3.3 Regulation of specific RNA stability

The *SOD1* gene is reported to interfere with RNA metabolisms, including microRNA (miRNA) biogenesis, mRNA transcription, splicing, transportation, and stabilization ³¹⁵. Previous studies found TDP43 and FUS proteins containing RNA binding motifs are chaperoned and imported into the nucleus, playing an important role in RNA processing and DNA damage repair ³¹⁶. Although SOD1 does not have RNA binding motifs, mutant *SOD1* stabilizes RNA metabolism through binding with mRNAs. For example, *HuR* and *VEGF* are two protective factors for motor neurons. Mutant *hSOD1* could impair the *HuR* function through binding with NFL-mRNA 3'-UTR (Neurofilament

Light Chain) and negatively affect its stability in motor neuron-like NSC34 cell lines ³¹¹. Besides, mutant *hSOD1* could also reduce the mRNA level of *VEGF* (Vascular Endothelial Growth Factor), which protects motor neurons from oxidative stress ³⁰¹. Mice with reduced *VEGF* gene expression exhibit similar phenotypes in patients with ALS ³¹⁷.

1.3.3.4 Regulation of cell signalling pathways

SOD1 plays a critical role in regulating cell signalling pathways by modulating the levels of ROS in cells. Its activity is tightly regulated, and dysregulation of SOD1 has been implicated in various pathological conditions, including neurodegenerative diseases, cancer, and aging.

One of the most critical pathways SOD1 regulates is the NF- κ B pathway, which is involved in inflammation, immune response, and cell survival. NF- κ B is activated by various stimuli, including ROS, which can contribute to the transcriptional regulation of genes implicated in these processes. By reducing the levels of ROS, SOD1 can inhibit the activation of NF- κ B, thereby reducing inflammation and promoting cell survival ³¹⁸. SOD1-G93A mice, however, have activated NF- κ B. Therefore, pharmaceuticals that inhibit NF- κ B activation may serve as a potential treatment for ALS ³¹⁹. The PI3K/Akt signaling pathway, which is involved in cell proliferation, survival, and metabolism, is also regulated by SOD1. SOD1 can activate this pathway by promoting the phosphorylation and activation of Akt, resulting in enhanced cell survival and proliferation ³²⁰. SOD1 also regulates the AMPK pathway and the Wnt signalling pathway ³²¹⁻³²³. By modulating these pathways, SOD1 can affect numerous cellular processes, such as proliferation, differentiation, and apoptosis. Overall, SOD1 is crucial in regulating cell signalling pathways by modulating ROS levels. Its activity is strictly regulated, and SOD1 dysregulation has been linked to several pathological conditions, including neurodegenerative diseases, cancer, and aging.

1.3.4 Impact of SOD1 in aging

1.3.4.1 Impact of oxidative stress on SOD1 protein

Oxidative stress exerts its impact by inducing various posttranslational modifications on proteins, primarily targeting sulfur-containing components such as the thiol group of cysteine and the thioether group of methionine. Protein is the primary target for oxidative stress, and its cysteine residues are essential for stabilizing protein structures. Cysteine is particularly vulnerable to oxidative modification because its sulfhydryl moiety is easily attacked by redox-active substrates such as glutathione or peroxides to form S-S or S-O covalent modification ¹³⁸. The first reaction by-product of a thiolate with H₂O₂ is sulfenic acid, which can then participate in various reactions depending on the circumstances. The sulfenic can form intermolecular or intramolecular disulfide bonds and be reversed by glutathione. Moreover, sulfenic can be further oxidized to sulfinic and sulfonic acids. This sulfonic acid is the most highly oxidized thiol species, and its formation is irreversible ³²⁴. SOD1 has four cysteine residues: Cys6, Cys57, Cys111 and Cys146. Since Cys111 is positioned on the edge dimer interface of each subunit, modifying Cys111 can disrupt the dimer contact at the stereochemical interface and induce SOD1 dissociation and is prone to oxidatively modified to sulfenic, sulfinic, or even sulfonic acid. The modification of Cys111 could make wild-type SOD1 prone to monomerization and decrease its enzymatic activity ³²⁵.

Nevertheless, Cys111 to serine (C111S) substitution is known to improve the structural stability and resistance to heat inactivation of wildtype SOD1, showing that the mechanism of Cys111 may affect the conformational state of SOD1 ³²⁶. The thiol group of Cys111 in SOD1 is oxidized to a sulfenic acid by hydrogen peroxide, and further dissociation of the associated metal ions from the oxidized SOD1 permits another free Cys6 to attack the sulfenylated Cys111 nucleophilically. Consequently, Cys6 and Cys111 create an intramolecular disulfide link. The aberrant SOD1 with

a non-canonical disulfide link was shown to be conformationally stretched, with considerable cytotoxicity and a high propensity to aggregate ³²⁷. Posttranslational changes of Cys111 are also known in mutant SOD1. A structural change in mutant SOD1 can increase the susceptibility of Cys111, located at the sulfhydryl moiety, to oxidative modification by substrates. This is due to the hindrance of Cys111 near the dimer interface. Glutathionylation of Cys111 is a common modification observed in mutant SOD1. Furthermore, peroxidized SOD1 at Cys111 has been observed in the inclusion bodies of G93A mutant SOD1 transgenic mice ³²⁸.

Although the exact nature of mutant SOD1 toxicity has yet to be determined, misfolding of mutant SOD1 protein is thought to play a significant role in the pathogenesis of familial ALS ³²⁹. There is a consensus that oxidative damage contributes to the misfolding of SOD1 and that oxidized proteins accumulate as cells age ²⁹⁹. The misfolded SOD1 is prone to dissociation into monomers, leading to aberrant binding, resulting in oligomers and, eventually, large molecular weight aggregates in cells. When proteins accumulate abnormally in neurons, it can disrupt their axonal transport and protein degradation machinery ^{330 331}. Patients with familial ALS and mutant SOD1 transgenic mice have been found to exhibit SOD1-positive inclusion bodies in their degenerating motor neurons in the spinal cords ³³².

When SOD1 is misfolded, copper or zinc bound to the SOD1 subunit is also at risk of being exposed to the tight dimer structure. Since copper is catalytically redox-active, aberrant chemical reactions can produce reactive oxygen or nitrogen species in addition to the initial SOD1 activity. When exposed to excess H₂O₂ in vitro, the enzyme undergoes selective oxidation of copper-coordinating residues. This leads to a loss of catalytic activity and oligomerization by mechanisms yet to be fully elucidated ³³³. Copper is linked to the zinc cofactor by ligating to His46, His48, His120, and His63. A further structural function in stabilizing the SOD1 homodimer is played by

zinc, which is coordinated to His71, His80, and Asp83³³⁴. An intra-subunit disulfide formed by Cys57 and Cys146 helps stabilize the enzyme and allows the histidine residues to coordinate with the metal cofactors more easily³³⁵. Therefore, either oxidation of a zinc ligand with loss of the metal or breakdown of the Cys57-Cys146 disulfide bond contribute to destabilization and aggregation of the hSOD1²⁹⁹.

1.3.4.2 Impact of SOD1 on mitochondrial function

Given that mitochondria are involved in generating oxidative stress and are vulnerable to reactive oxygen species (ROS), it is reasonable to hypothesize that they represent a primary site for SOD1 oxidation³³⁶. Mutant SOD1 monomers form disulfide-linked oligomeric clusters when they enter the IMS via the general import pore to form protein aggregation, which induces mitochondrial ETC malfunction, membrane potential loss, and mitochondrial damage³³⁷. Studies have proved mutant SOD1 localized to the IMS enhanced cellular sensitivity to oxidative stress in NSC34 cells³³⁸. Muscle atrophy and neurodegeneration resulted from *in vivo* expression of mutant SOD1 localized to the mitochondrial IMS³³⁹. Moreover, mitochondria extracted from the spinal cords of G93A animals showed higher dismutase activity than mitochondria from WT, which was associated with higher ROS generation³⁴⁰. Hence, presymptomatic G93A rat mitochondria showed enhanced state IV respiration and H₂O₂ generation³⁴¹. This is consistent with the increased metabolism seen in ALS patients³⁴².

Mitochondria are negatively impacted not only by the presence of mutant SOD1 in the IMS but also by the presence of mutant protein on the outer membrane. Many studies found that mitochondria in the spinal cord had misfolded SOD1 accumulated on its outer mitochondrial membrane³⁴³. The buildup of misfolded SOD1 on the outer mitochondrial membrane (OMM) has been linked to impairments in mitochondrial function by disrupting ion conductance through the

voltage-gated ion channel (VDAC) ³⁴⁴. Mutant SOD1 binding to Bcl2 has been demonstrated to promote the mitochondrial association of SOD1, which may weaken the role of Bcl2 as an anti-apoptotic regulator ³⁴⁵. Nevertheless, contrary to the liver, mutant SOD1 was found to associate with mitochondria in the central nervous system (CNS), notably in the spinal cord ^{296,346}. Following this finding, the cytosolic macrophage migration inhibitory factor (MIF) was shown to block the association of SOD1 with mitochondria and ER in healthy liver and other tissues. The low MIF levels in motor neurons may explain why mitochondria are more susceptible than different types of cells ³⁴⁷.

1.3.4.3 Impact of SOD1 on aging

Early findings point to diverse influences of SOD1 in invertebrate aging. Although having essentially normal phenotypic and health throughout development and early adulthood, researchers show that SOD1^{-/-} mice had a shorter lifespan than wild-type mice ³⁴⁸. As early as 3 months of age, the loss of SOD1 causes multiple episodes of elevated oxidative stress over an animal's lifetime and results in the buildup of senescent cells, which leads to increased inflammation and induces severe oxidative damage in multiple organs such as the gut, liver, ovary, and testis ^{348,349}. Besides, studies have found that senolytics (dasatinib and quercetin) could reduce cellular senescence in SOD1 knockout mice ³⁵⁰. To a significant degree, neuromuscular abnormalities in aged WT mice can also be observed in SOD1 KO animals. However, deletion of SOD1, particularly in neurons, causes enhanced loss of muscle mass and force only in elderly (24-29 month-old) mice, suggesting that severe muscle reductions need the accumulation of age-related alterations to the threshold where structural and functional maintenance is no longer achievable ³⁵¹.

Overexpression of SOD1 in *C. elegans* has been proven to extend the worm's lifespan not by scavenging O₂• but by stimulating transcription factors that promote longevity³⁵². However, it was reported to be deleterious and accelerated the aging process in mouse models. The hSOD1^{WT} transgenic mice show multiple neurodegenerative changes, including (1) mitochondrial swelling and vacuolization, especially in the spinal cord, brain stem, and subiculum axons; (2) axonal degeneration in several long fiber tracts, especially the spinocerebellar tracts; and (3) a moderate loss of spinal motoneurons by the age of 2 years³⁵³. With mild motor impairments and the onset of neurodegenerative alterations, hSOD1 transgenic mice are also proven in higher levels of mitochondrial DNA deletion, an indicator of aging, when they are compared to non-transgenic mice³⁵⁴.

1.3.5 The gap of knowledge between SOD1 and aging

In recent decades, the “Oxidative Stress Theory of Aging” has risen to prominence as a leading explanation for the biological and molecular mechanisms that underlie the aging process. This theory is substantiated by a substantial body of evidence. There are various perspectives regarding the impact of oxidative stress on the aging process. On one hand, extensive data demonstrates a notable increase in oxidative damage to lipids, DNA, and proteins as individuals age, across various tissues and animal models³⁵⁵. On the other hand, studies involving animal models reveal that longer-lived animals tend to exhibit reduced oxidative damage and enhanced resistance to oxidative stress³⁵⁶. It is suggested that oxidative stress may have effects beyond damage during aging. Therefore, a knowledge gap persists concerning the potential influence of oxidative stress contributing to the process of aging.

ROS play a role in various oxidative reactions. Most oxidative alterations on proteins are associated with protein dysfunction, either because of a direct modification in a domain needed

for protein function or because of the partial unfolding of the protein ³⁵⁷. As proteins lose their three-dimensional structure and undergo modifications to amino acid side chains and the protein backbone, the inner hydrophobic core structure interacts with different hydrophobic groups, leading to protein aggregation ^{358,359}. A robust connection exists between protein aggregation and the aging process, establishing a reciprocal relationship: protein modifications, oxidations, and the formation of cross-linked aggregates seem to contribute to the initiation and acceleration of the aging process ³⁶⁰. Furthermore, it is evident that the cytotoxicity of protein aggregates is implicated in age-related disorders, such as neurodegenerative diseases ³⁶¹, cancer ³⁶², and diabetes ³⁶³. Notably, SOD1 is particularly susceptible to such oxidative modifications under oxidative stress ³⁰². These protein alterations have been directly linked to both familial and sporadic ALS. Highly oxidized SOD1 proteins have been identified in potentially harmful protein aggregates and amyloid fibrils ³⁶⁴. However, the precise molecular mechanisms and potential consequences of oxidized SOD1-induced cytotoxicity in the context of aging remain poorly understood.

A noteworthy feature observed in ALS patients is the existence of SOD1-positive inclusion bodies within deteriorating motor neurons. These inclusion bodies may serve as precursors to mitochondrial dysfunction, ultimately preceding the degradation of motor neurons ³⁶⁵. Although soluble misfolded/soluble total SOD1 ratio highly existed in most affected regions, however, a study proved a higher amount of SOD1-positive aggregates in the spinal cord could prolong the lifespan of mutant SOD1 mice due to its ability to bind and store harmful species²⁹⁶. Therefore, the available evidence is insufficient to conclusively determine whether SOD1 exerts a positive or negative impact on the aging process, necessitating further investigation.

1.4 Summary

Considering the intricately complex changes, the aging process is marked by a multifaceted decline in functionality. The accumulation of oxidized SOD1 within mitochondria has been implicated in instigating declines in mitochondrial function ³⁶⁶. However, the mitochondrial function does not necessarily follow a linear decline throughout the aging process due to the existence of mitochondrial quality control mechanisms. Middle-aged individuals exhibit a compensatory increase in the removal of damaged mitochondria and oxidized proteins, but mitochondrial dysfunctions become more pronounced in advanced age ³⁶⁷.

SOD1 mutations have been established as a contributing factor to increased vulnerability to oxidative stress ³⁶⁸. A notable characteristic of ALS patients is the presence of SOD1-positive inclusion bodies within deteriorating motor neurons, which may act as precursors to mitochondrial dysfunction, ultimately preceding motor neuron degradation ³⁶⁹. However, the precise role of hSOD1 oxidation in the aging process remains unclear. To dispose of the oxidized proteins, MDVs have been found to serve as transporters ²²⁹. Nevertheless, the fate of MDVs originating from mitochondria remains a subject of debate, with the possibility of their degradation within lysosomes or secretion into the extracellular space, potentially affecting paracrine signaling. Therefore, this research aims to address the research questions: Is oxidized hSOD1 a causal factor of aging and does the MDV modulation represent one of the underlying mechanisms? Understanding the intricacies of MDV-mediated secretion sheds light on the mechanism of aging. Consequently, our study aims to elucidate the role of oxidized hSOD1 in aging, to explore the molecular targets and to modulate mechanisms of MDVs at an early stage, holding promise as a potential therapeutic approach for age-associated disorders. Such approaches could provide valuable insights into the nature of aging.

Chapter II

Oxidatively modified SOD1 is a causing factor of aging

2.1 Introduction

Aging is marked by the onset of cellular senescence and the gradual decline of their function. Even though ROS mechanisms in aging are not fully understood, mounting evidence supports ROS as a significant contributing factor in the aging process³⁷⁰. Endogenous reactive oxygen species (ROS) play a crucial role in regulating various cellular signaling pathways, whereas excessive production of ROS at elevated levels can result in their interaction with multiple biomolecules, such as lipid membranes, proteins, and nucleotides. This can accelerate the process of cell senescence and ultimately lead to cell death. The "oxidative stress theory" suggests that ROS can lead to oxidative damage, which deteriorates physiological function, increases disease risk, and reduces life expectancy⁸⁰.

SOD1 is an important antioxidant enzyme that regulates the baseline levels of superoxide and has been known for the catalysis of superoxide to hydrogen peroxide for nearly half a century³⁰⁹. SOD1 is primarily localized in the cytoplasm but also the nucleus³⁰⁹, mitochondrial inner membrane space³⁷¹, and peroxisomes³⁷². SOD1 is initially present in the cell as an inactive apo-monomer, which requires post-translational modifications (PTMs) to become active. These modifications include binding to zinc for structural stability, acquiring copper for enzymatic activity, and forming an intramolecular disulfide bond between Cys57 and Cys146³⁷³. There are additional PTMs in SOD1, including phosphorylation, glycation, palmitoylation, and acetylation³¹¹. SOD1 exhibits other cytoprotective activities against ROS, including gene transcription in response to oxidative stress and involvement in signal transduction pathways associated with ROS²⁹⁸. If knockdown of the SOD1, the accumulation of oxidants accelerates cellular senescence³⁷⁴. As a result, SOD1^{-/-} mice lose their muscle mass and increase oxidative damage with age³⁴⁸.

The discovery of the first SOD1 gene mutations in a small cohort of familial ALS patients in 1993 marked a watershed moment in SOD1 biology ³⁷⁵. This discovery shifted the focus from a basic understanding of the physiological functions of SOD1 to recognizing its involvement as a contributing factor in age-related diseases and neurodegeneration. SOD1 is involved in a variety of molecular processes of cytotoxicity, many of which do not merely result from the loss of its cytoprotective capabilities ³⁷⁶. Several redox alterations may occur under normal physiological circumstances and oxidative stress. As previously stated, reactive stress can modify the side chains and the peptide links of proteins ³⁷⁷. Although PTMs of SOD1 stabilize protein structure and activate antioxidant function through correctly folding from monomer to dimers, redox regulations may allow protein cysteine thiols to sustain and withstand irreversible and reversible PTM under oxidative stress ³⁷⁸. SOD1 mutations are thought to contribute to pathology in a variety of disorders via several processes, including ALS ³⁷⁹, PD³⁸⁰, heart failure ³⁸¹, cancer ³⁸², and diabetes ³⁸³. Misfolded SOD1 adopts an oxidatively modified conformation exposing its hydrophobic sequences, which are covalently attached to exposed hydrophobic patches on other misfolded SOD1 to form oligomers. These oligomers can even be covalently attached to native proteins and create high molecular weight aggregates ³⁸⁴. When oxidative modification leads to misfolding, oligomerization, or aggregation, SOD1 gains toxicity ³⁸⁵. Protein cysteine thiols (-SH) are a target of ROS and modulators such as glutaredoxins and glutathione transferases ³⁸⁶. It may create a sequence of changes in their oxidation state, which can determine the destiny of specific proteins. The Cys111 has been identified as a crucial residue in controlling SOD1 stability and dimerization in response to oxidative stress and aging ³⁸⁷. However, the Cys111 of hSOD1 is more vulnerable to oxidative modification than other cysteine residues because of its proximity to the dimeric interface ²⁹⁶. Therefore, Cys111 has emerged as a residue of particular interest in unraveling the

mystery of toxicity of hSOD1 in many diseases. Mutant yeast SOD1 was employed by Bastow et al. under age-related circumstances to confirm that yeast SOD1 does not aggregate³⁸⁸. Because there is no Cys111 in ySOD1, lending credence to the hypothesis that this residue may play a role in the development of hazardous hSOD1 aggregates³¹¹. As such, oxidation of SOD1 at Cys111 may contribute to age-related diseases by initiating the oxidation of SOD1.

Motor neurons are specialized nerve cells that transmit signals from the brain to the muscles, enabling voluntary movements. They form the primary component of the motor system. In some cases of familial ALS, mutations in the *SOD1* gene can produce a mutant form of hSOD1 protein prone to misfolding and aggregation³⁸⁹. These aggregates, often referred to as "oxidized SOD1," can accumulate within motor neurons and contribute to their dysfunction and degeneration. The exact mechanisms by which oxidized SOD1 leads to motor neuron death are still not fully understood, but it is believed to involve a combination of toxic gain-of-function properties and impaired cellular processes³⁹⁰. The accumulation of oxidized SOD1 is thought to disrupt normal cellular functions, leading to mitochondrial dysfunction, excitotoxicity (excessive activation of specific receptors), impaired protein degradation systems, and activation of inflammatory processes in motor neurons³⁹¹. However, the specific role of oxidized SOD1 in motor neuron senescence remains incompletely understood and necessitates further investigation.

Neural stem cells (NSCs) are responsible for generating and replenishing neurons and glial cells in the central nerve system. In the mature central nervous system, cells with neural stem cell characteristics are primarily localized to two key regions: the subventricular zone and the subgranular layer of the dentate gyrus within the hippocampal formation. NSCs display various dynamic behaviors throughout their lifecycle, including quiescence, activation, proliferation, and migration. NSCs can enter a quiescent or dormant state, where they remain in a non-dividing,

inactive state. This quiescent phase allows NSCs to maintain their stem cell pool and preserve their long-term regenerative capacity. NSCs can be stimulated to transition from a quiescent state to an active state. Various intrinsic and extrinsic factors, such as growth factors, neurotransmitters, and environmental cues, can trigger the activation of NSCs ³⁹². Once activated, NSCs undergo proliferation, resulting in the expansion of their population. To fulfil their roles in central nerve system, NSCs must maintain cellular integrity and functionality over extended periods. However, as individuals age, the regenerative capacity of neural stem cells diminishes, leading to a decline in their ability to produce new neurons. This age-related decline in neural stem cell function can contribute to various aspects of aging in the brain, including reduced cognitive function ³⁹³, impaired learning and memory ³⁹⁴, and an increased susceptibility to neurodegenerative diseases³⁹⁵. Proteostasis mechanisms of NSCs are crucial in supporting self-renewal and differentiation capabilities ³⁹⁶. One aspect of proteostasis relevant to NSCs is the regulation of protein folding and quality control. NSCs are exposed to various stresses during their lifetime, such as oxidative stress, DNA damage, and environmental insults. These stresses can lead to the accumulation of misfolded or damaged proteins. Although the specific impact of oxidized SOD1 on NSCs is not well understood, oxidative stress in general can negatively affect cellular processes, including NSC function ³⁹⁷. High levels of oxidative stress can disrupt cellular integrity and functionality, leading to impaired NSC self-renewal and differentiation abilities ³⁹⁸. NSCs rely on molecular chaperones and protein quality control systems, such as ubiquitin-proteasome and autophagy, to ensure proper protein folding and eliminate aberrant proteins ³⁹⁹. Failure in proteostasis can impair NSC function and contribute to age-related decline or neurodegenerative disorders ⁴⁰⁰. As organisms age, NSCs undergo changes that can affect their proteostasis mechanisms, leading to cellular dysfunction and impairments in neurogenesis ⁴⁰¹.

Research is currently underway to investigate the role of oxidized SOD1 in the aging process and how manipulating proteostasis pathways can promote healthy aging. However, further research is needed to comprehend the underlying mechanisms. Additionally, exploring potential therapeutic interventions to reduce the accumulation of oxidized proteins during aging is crucial. This avenue of investigation holds promise for developing strategies that could enhance overall health and well-being throughout aging.

2.2 Materials and Methods

2.2.1 Cell culture and transfection

Mouse motor neuron (NSC34) cells were purchased from CELLutions Biosystems Inc. (Catalogue: CLU140, Ontario, Canada). Cells were cultured as described in the previous manuscript⁴⁰². Briefly, NSC34 cells were seeded at 1×10^6 cells/ml in 2 mL of DMEM (11960069, Gibco, Waltham, USA) culture medium at 37 °C in a humidified incubator with 5% CO₂. The DMEM culture medium was enriched with 10% fetal bovine serum and 1% penicillin. When grown at 60-70% confluent, cells were transfected with either an empty plasmid, hSOD1^{G93A} or hSOD1^{WT} plasmid (500ng total DNA/2 x 10⁵ cells) with lipofectamine 2000 (11668, Invitrogen, Waltham, USA) for 48 hours. Fresh media supplemented with G418 Sulfate Solution (MIR 5920, Mirus Bio, Madison, USA) at a concentration of 0.4mg/mL was added to select transfected cells, while non-transfected cells, as a control, were dead within one week. Cells with more than 70% confluence were transferred into a T25 flask supplemented with 0.4mg/mL of G418 and cultured for a total of three weeks for further experiments. The positively transfected cells were identified by anti-hSOD1 antibody (ab52950, Abcam, Waltham, USA) by Western blot.

2.2.2 Primary neural stem cell culture and differentiation

Transgenic mouse strains carrying the human G93A mutant SOD1 (B6.Cg-Tg(SOD1*G93A)1Gur/J; 004435) and wild-type human SOD1 (B6.Cg-Tg(SOD1)2Gur/J; 002298) were obtained from the Jackson Laboratory (Bar Harbor, USA). These transgenic lines were maintained by breeding hemizygous carrier males with female mice possessing a C57BL/6 background. The colonies of these mouse strains are currently housed at the Central Animal Care Services, University of Manitoba, and all experimental procedures involving the mice were conducted in strict accordance with the guidelines outlined in the Guide of Care and Use of Experimental Animals of the Canadian Council on Animal Care. Following a previously described protocol in our lab⁴⁰³, female C57BL/6 mice were paired with transgenic male mice carrying either the full-length wild-type human SOD1 (hSOD1^{WT}) or the G93A mutation of human SOD1 (hSOD1^{G93A}) gene. As a positive vaginal plug was considered to have occurred on day E0.5 of pregnancy, E14.5 pregnant mice was used as the reference point and were euthanized humanely using carbon dioxide asphyxiation followed by cervical dislocation. The embryos were carefully collected and genotyped by using DNA from mouse tails. Cortices were removed from embryonic brains, triturated, and then strained through a 40 M sieve. After harvesting the cells, we cultivated them for 5–7 days in neurosphere-forming Medium (NSC SFM) (A1050901, ThermoFisher, Waltham, USA) to promote neurosphere development. Neurospheres were detached for culturing after their diameter reached 150-200 µm. The NSCs were extracted by centrifugation and deposited onto 6-well plates coated with poly-L-ornithine and laminin using Complete StemPro NSC SFM (A1033201, ThermoFisher, Waltham, USA) for the differentiation process. To obtain enriched differentiated neurons, the culture medium was changed to neurobasal/B27 (A3653401, ThermoFisher, Waltham, USA), and anti-MAP-2 (ab32454, Abcam, Waltham, USA) immunostaining was used to confirm neuronal differentiation.

2.2.3 Plasmid Construct

The hSOD1-G93A and wild-type genes were cloned from total RNA extracted from the transgenic mice carrying hSOD1-G93A or hSOD1-WT. The G93A template for PCR was amplified with the following primers: 5'-CTGCTGACAAAGATGCTGTGGCCGATGTGTC-3' (forward), 5'-GACACATCGGCCACAGCATCTTTGTCAGCAG-3' (reverse). The WT template uses the following primers: 5'-GCGCGCGTCGACAAGCATGGC-3' (forward), 5'-GCGCGCGTCGACGCTTGGGGCGATCCCAAT-3' (reverse). Automated sequencing was used to verify all plasmid constructions.

2.2.4 EdU assay staining

Cells were seeded in a 24-well plate at 5×10^4 cells/ml in 500 μ L of DMEM culture medium overnight. Replace 250 μ L of culture medium with 250 μ L of $2 \times$ Working solution of the EdU (250 μ M, MAN0002026, Invitrogen, Carlsbad, USA) to each well at 37 °C for 2 hours, as previously described⁴⁰⁴. Remove the medium and fix the cells with 4% paraformaldehyde for 15 min. Rinse the cells with 3% BSA 3 times (5min each time). Permeabilize the cells with 0.25% Triton X-100 in PBS (PBSTx) for 20 min. Remove the PBSTx and wash twice with 3% BSA 3 times. Remove the wash solution and add 200 μ L/well reaction cocktail and rock the plate to distribute the solution evenly. Incubate for 3 min and escape from the light. Remove the reaction cocktail and rinse once with 3% BSA. Then remove the BSA and incubate the samples with 200 μ L/well anti-hSOD1 primary antibody (1:1000, ab52950, Abcam, Waltham, USA) overnight at 4°C. After washing with PBS, the secondary antibody in 1% BSA/PBSTx were incubated at room temperature in the dark for 1 hour. Discard the antibody and wash it with PBS once. The nuclei were stained with Hoechst33258 (94403, Sigma, Virginia Beach, USA) for 5 min and then washed with PBS three times. Finally, mount the coverslips with 6 μ L of fluorescent mounting medium

on slides (S3023, Dako, California, USA). Dry the slides for 1 hour and observe under the Z1 microscope.

2.2.5 SA- β -gal staining

Cells were seeded in a 24-well plate at 5×10^4 cells/ml in 500 μ L of DMEM culture medium overnight. Remove the culture medium and rinse the plate with PBS once. Add 200 μ L fixative solution (11674, Cell Signaling Technology, Beverly, USA) to fix the cells for 15 min at room temperature. Rinse the plate with PBS twice. Add 1mL β -Galactosidase Staining Solution (9860, Cell Signaling Technology, Beverly, USA) to each well and incubate at 37 °C in a dry incubator without CO₂ for 48 h, as previously described ²⁷⁴. Check the cells under a microscope with a magnification of 200 $\times$ to verify the development of blue color in the cells.

2.2.6 Thiol oxidation of hSOD1 detection

The thiol group of hSOD1 and total hSOD1 were measured by the Human Cu/ZnSOD Antibody Pair ELISA kit (BMS222MST, Invitrogen, Waltham, USA) according to the manufacturer's protocol. Briefly, 10mL coating antibody was pre-prepared with 250 μ L coating antibody and 9750 μ L PBS. 100 μ L coating antibody was added into each well overnight at 4°C. The washing buffer was applied to wash the plate once for 15 s. The plate was blocked with 250 μ L assay buffer (5g BSA in 1 L washing buffer) overnight at 4°C. 100 μ L samples and 500 μ L Maleimide Activated Horseradish Peroxidase (31485, Thermo Fisher Scientific, Waltham, USA) were added into each well and incubated for 20 min on a microplate shaker. The plate was washed twice with washing buffer (10-15 s for each time). Then PBS and standard proteins were added into each well, and the plate was covered with an adhesive film at room temperature on a microplate shaker for 1 h. The plate was washed with washing buffer 3 times. 100 μ L TMB Substrate Solution was added into each well immediately. The color development on the plate was monitored. Then 100 μ L stop

solution was added to stop the color development. The absorbance of each microwell was read on a spectrophotometer using 450 nm as the primary wavelength.

2.2.7 Soluble and insoluble hSOD1 detection

As previously described in our lab ⁴⁰⁵, differential extractions were employed to evaluate SOD1 levels. Briefly, cells were collected into a pellet, which was then washed with PBS. The cell pellet was subsequently resuspended in 100 μ L of 1 $\times$ TEN buffer (consisting of 10 mM Tris, 1 mM EDTA, and 100 mM NaCl). An equal volume of extraction buffer A (1 $\times$ TEN, 1% Nonidet P-40, and 1% protease inhibitor cocktail) was added to the cell suspension, followed by intermittent sonication for 30 seconds. The resulting lysate was then centrifuged at 100,000 g for 5 minutes using an ultracentrifuge (Optimal L-90k, Beckman Coulter, Inc., Brea, USA) to separate the P1 pellet from the S1 supernatant. The S1 supernatant, containing soluble SOD1, was stored on ice for further analysis. Next, the P1 pellet was subjected to an additional round of sonication with 200 μ L of extraction buffer B (1 $\times$ TEN and 0.5% Nonidet P-40) for 30 seconds. Subsequently, the lysate was centrifuged again at the same speed and duration as before to collect the P2 pellet, containing insoluble SOD1. The P2 pellet was resuspended in buffer C (1 $\times$ TEN, 0.5% Nonidet P-40, 0.25% SDS, and 0.5% deoxycholic acid) and sonicated for 30 seconds. Finally, the protein concentration of the S1 and P2 samples was determined using the BCA Protein Assay Kit (23227, Thermo Fisher Scientific, Waltham, USA).

2.2.8 CT4-GST pull-down assay

The CT4-GST pull-down assay is a specific variation of the GST pull-down technique that is used to isolate and study the interactions of misfolded SOD1 protein selectively. Briefly, the pGEX-4T-1 vector containing the derlin1-CT4 epitope (FLYRWLPSRRGG) was transformed into BL21 competent cells. A clone was selected and cultured in LB media supplemented with 50 μ g/mL

ampicillin. Expression of either GST alone or GST-fused GGGGS-CT4 was induced at an OD₆₀₀ of approximately 0.6 using 0.5 mM IPTG. The bacterial pellet was resuspended in lysis buffer (30 mM Tris-Cl, pH 7.5, 0.1 mM NaCl, 1 mM DTT, 1% NP-40, and protease inhibitors), sonicated, and then centrifuged at 12,000 rpm for 30 min. The soluble fraction containing GST or GST-GGGGS-CT4 fusion proteins was incubated with glutathione-Sepharose 4B beads (Cytiva, Marlborough, USA) for 1 h to immobilize the proteins. To investigate the direct binding between misfolded hSOD1 and CT4, the immobilized GST-fused proteins were incubated with the samples at 4°C for 2 h. After washing the beads three times with PBS, the bound proteins were separated by SDS-PAGE and detected through immunoblotting. This experimental setup allowed for the identification of potential interactions between misfolded hSOD1 and CT4. The binding between these proteins was examined by analyzing the presence of specific bands on the immunoblot, indicating direct protein-protein interaction.

2.2.9 Extracellular vesicle (EV) isolation and labelling

The culture medium of NSCs was collected every other day and centrifuged at 1500 g for 10 minutes at 4°C, followed by 10,000 g for 30 minutes at 4°C. The resulting medium was subsequently filtered with 0.22 µm filters. EVs were isolated using the ExoQuick-TC reagent (SBI, Mountain View, USA) as previously described⁴⁰⁶. Transmission electron microscopy (TEM) was used to corroborate the EVs. To label the EVs and monitor their internalization, EVs were transfected with Texas-red labelled RNA oligonucleotides using the Exo-Fect exosome transfection reagent (System Bioscience, Palo Alto, USA) according to the manufacturer's instructions.

2.2.10 Western blot

Mitochondria and MDVs were homogenized with the RIPA lysis buffer (89900, Thermo Fisher Scientific, Waltham, USA), as previously described⁴⁰⁷. The protein concentrations were measured by using a Pierce BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, USA). Afterwards, the homogenates were electrophoretically separated using SDS-PAGE. Proteins were separated by electrophoresis and transferred to PVDF membranes, where they were blocked in the blocking buffer (5% milk in PBST) for an hour. A primary antibody was then incubated overnight at 4°C on the membrane, followed by a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. An ECL kit was used to visualize the antigen-antibody complexes (1705061, Bio-Rad). Stain-free total protein was applied as loading control of total proteins⁴⁰⁸⁻⁴¹⁰. Histone H1 was used as nuclear control and β -actin as cytoplasmic control.

2.2.11 Immunofluorescence staining

Coverslips were seeded with cultured cells at 5×10^4 cells/ml in 500 μ L of DMEM culture medium, then fixed for 15 min at room temperature with 4% paraformaldehyde as previously described⁴¹¹. After permeabilization with 0.25% Triton X-100 in PBS for 10 min, cells were blocked with 1% bovine serum albumin in PBST for 30 min. The primary antibody was first added to the samples and incubated overnight at 4 °C. After washing with PBS, the secondary antibody in 1% BSA/PBST were incubated at room temperature for 1 h. Discard the antibody and wash the slides. Nuclei were stained with Hoechst 33258 (94403, Sigma, Virginia Beach, USA) for 5 min, and then mounting the coverslip with a drop of fluorescent mounting medium (S3023, Dako, Carpinteria, USA). Store the slides in the dark at -20°C.

Table 2 Primary Antibodies

Antibody	Cat#	Host	Application	Dilution	Company
A5C3	MM0070-3-P	Mouse	WB	1:500	Medimabs, Montreal, Canada
p53	ab26	Mouse	WB	1:1000	Abcam, Waltham, USA
p16 ^{INK4A}	sc166760	Mouse	WB	1:1000	Santa Cruz, Dallas, USA
Mitofusin 2	ab56889	Mouse	WB	1:1000	Abcam, Waltham, USA
PGC-1 α	ab54481	Rabbit	WB	1:1000	Abcam, Waltham, USA
HMGB1	ab228624	Rabbit	WB; IF	1:1000	Abcam, Waltham, USA
hSOD1	ab52950	Rabbit	WB; IF	1:1000	Abcam, Waltham, USA
8B10	MA1-105	Mouse	WB	1:2000	Invitrogen, Carlsbad, USA
Syntaxin 17	ab229646	Rabbit	WB	1:500	Abcam, Waltham, USA
β -actin	sc47778	Mouse	WB	1:5000	Santa Cruz, Dallas, USA
Histone H1	sc8030	Mouse	WB	1:1000	Santa Cruz, Dallas, USA
MAP2	sc-20172	Rabbit	IF	1:2000	Santa Cruz, Dallas, USA
SOX2	ab79351	Mouse	IF	1:1000	Abcam, Waltham, USA
B8H10	MM-0070-P	Mouse	IF	1:500	Medimabs, Montreal, Canada
Ki67	9129S	Rabbit	IF	1:1000	NEB, Ipswich, USA
Nestin	sc-33677	Mouse	IF	1:2000	Santa Cruz, Dallas, USA

2.2.12 Transmission electron microscopy (TEM)

When hSOD1^{WT} and hSOD1^{G93A} NSC34 cells grew to 70-90% confluent in the T25 flasks, cells were washed with pre-warmed PBS. 1ml trypsin was added to the flask at room temperature for around 1min. 5 mL culture medium was added to the flask, using a pipette to detach the cells from

the surface of the flask. Suspended cells were Collected in a 15 mL centrifuge tube, and the suspension was centrifuged at 100 g for 5 min. Then the supernatant was removed, the pellet was washed twice, and the cell pellet was transferred to a 2 mL centrifuge tube. The pellet was resuspended by 1.5 mL of 3% glutaraldehyde solution as previously described ⁵¹. The tube was placed in a shaker for 2 hours at room temperature. The cell pellet was rinsed by 3 times with a 6% sucrose solution. The samples were incubated in the buffer on the shaker for 15 min each time. After removing the sucrose, the samples were post-fixed in 1% Osmium tetroxide O_8O_4 (19152, Electron Microscopy Sciences) in 0.1M Sorensen's buffer for 2 h. Then, O_8O_4 was removed and discarded in a waste container. 0.1M, 0.05 M Sorensen's buffer was applied to rinse three times, and ddH₂O was applied for 10 min each time. Samples were dehydrated with 30%, 50%, 70%, 95%, and 100% Ethanol (15058, Electron Microscopy Sciences) and 100% Methanol separately for a total of 120 min. Then the samples were embedded with 100% propylene oxide (20401, Electron Microscopy Sciences) for 20 min, with 1:2 Epon and 2:1 Epon for 1 h separately, and with 100% Epon overnight. The 100% Epon was prepared by mixing the reagents bought from Electron Microscopy Sciences, including EMBed-812 (14900, Electron Microscopy Sciences), DDSA (13710, Electron Microscopy Sciences), NMA (19000, Electron Microscopy Sciences), and DMP-30 (13600, Electron Microscopy Sciences). The samples were placed in desired models filled with 100% Epon and placed in an oven heated at 60 °C for 24-48 h. The samples were trimmed into thin sections with 90 µm by microtome. Thin sections were then collected onto copper grids and stained with UranylLess (22409, Electron Microscopy Sciences) for 3 min and lead citrate (22410, Electron Microscopy Sciences) for 1 min, followed by 3 times washing in water. Finally, the photography was taken by using a Philips CM10 electron microscope at 60 kV.

2.2.13 Detection of Protein Carbonyl Content

Protein carbonyl content assay (ab126287, Abcam) was performed according to the manufacturer's instructions to evaluate the antioxidant capacity in the homogenized brain tissues as previously described ⁴¹². Briefly, samples were dissolved in dH₂O and diluted to approximately 10 mg/ml protein. 100 μ L DNPH was added to each sample. The mixture was incubated for 10 min at room temperature while vortexing. 30 μ L of TCA was added to each sample, vortexed, and placed on ice for 5 min. After that, samples were spined at maximum speed for 2 min. The supernatant was removed and discarded without disturbing the pellet. 500 μ L of cold acetone was added to each tube to wash the pellet. Thirty seconds in a sonicating bath was typically sufficient to disperse the pellets effectively. The samples were placed at -20°C for 5 min and then centrifuged for 2 min. Then, acetone was carefully removed. 200 μ L of Guanidine solution and sonicate were added. 100 μ L of each sample was transferred to the 96-well plate. The OD value was measured at ~375 nm in a microplate reader.

2.2.14 Detection of malondialdehyde (MDA)

The lipid peroxidation, measured through monitoring malondialdehyde (MDA), was determined using the Thiobarbituric Acid Reactive Substances (TBARS) kit (KA1381, Abnova) ⁴¹³. Briefly, 100 μ L of samples or the standard was added to labelled 5mL vials. 100 μ L of SDS solution was added to the vial and swirled to mix. 4mL of the Color Reagent was added downside of each vial forcefully. Vials were boiled for one hour. After one hour, the vials were immediately removed from the boiling water and placed in an ice bath for 10 minutes to stop the reaction. Then, the vials were centrifuged for 10 minutes at $1,600 \times g$ at 4°C. 150 μ L (in duplicate) of the reagents from each vial was loaded to the clear plate (colorimetric version). Read the absorbance at 530-540 nm at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.

2.2.15 Statistical analysis

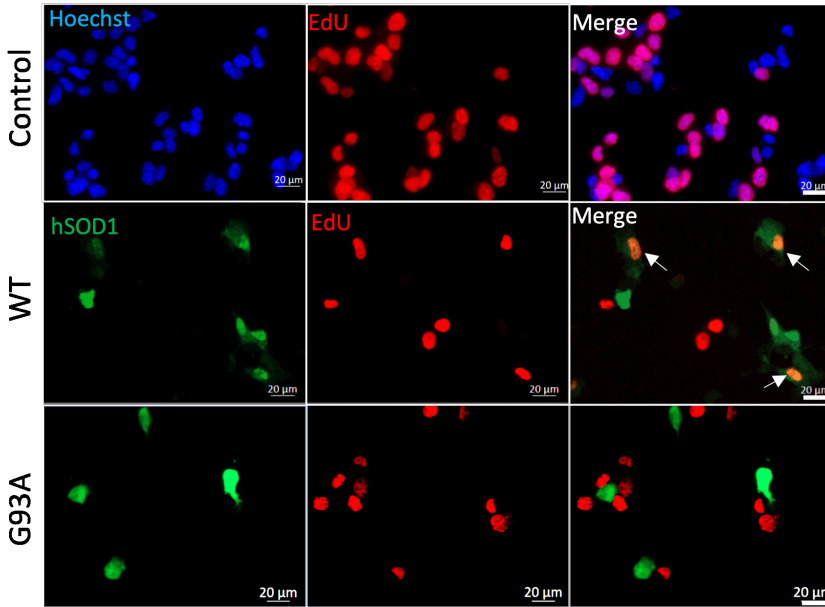
Statistical analysis of the data was performed using Prism 9. Data were expressed as mean $\pm$ standard error of the mean (SEM). An unpaired t-test was applied to compare differences between the control and experimental groups. Multiple comparisons were performed by one-way ANOVA test followed by Bonferroni's test or two-way ANOVA followed by Dunn-Sidak's test. Differences were considered statistically significant when * $p < 0.05$, ** $p < 0.01$.

2.3 Results

2.3.1 NSC34 cells expressing the hSOD1^{G93A} undergo early entrance into senescence

Cellular senescence is one of the hallmarks of aging characterized by irreversible growth arrest, the accumulation of β -galactosidase, and the release of proinflammatory factors. To investigate the role of hSOD1 in cellular senescence, we transfected hSOD1^{G93A} and hSOD1^{WT} plasmids to NSC34 cells for 48 hours and cultured the cells for 3 weeks. Cells from each week were performed with EdU assay and stained with anti-hSOD1 antibody (Fig.2.1a). The colocalized cells indicate proliferative cells with positive hSOD1 transfection. Data revealed a persistent low proliferative rate in hSOD1^{G93A} transfected NSC34 (NSC34^{G93A}) cells from 7.67% after transfection for 48 hours to 9.91% for 3 weeks. However, the proliferative rate in hSOD1^{WT} transfected NSC34 (NSC34^{WT}) cells increased from 8.38% after transfection for 48 hours to 23.83% for 3 weeks. Besides, the proliferative cells in NSC34^{WT} cells were 2.4-fold higher than that in hSOD1^{G93A} cells in the 3rd week (Fig.2.1b). The result indicates hSOD1^{G93A} NSC34 cells undergo premature senescence characterized by growth arrest.

(a)



(b)

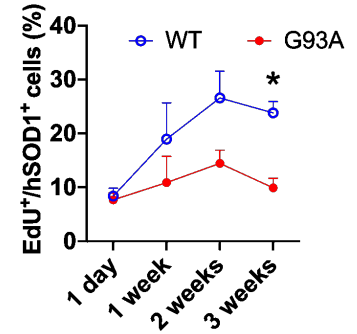
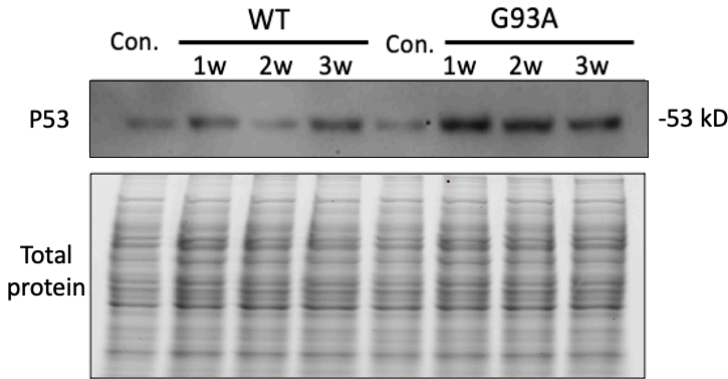


Fig.2.1 NSC34^{G93A} cells undergo premature senescence characterized by growth arrest

(a) Detection of EdU⁺ (red) in non-transfected (hSOD1⁻), hSOD1^{WT} and hSOD1^{G93A} transfected (hSOD1⁺, green) NSC34 cells. Scale bar, 20 μ m. (b) Quantification of the proportion of EdU⁺ cells in NSC34 cells expressing hSOD1 within 3 weeks (n=20-50 cells), * $p < 0.05$.

P53 gene acts as a central player in activating and maintaining cellular senescence, exerting its influence through multiple downstream targets and pathways to ensure the proper response to cellular stress and maintain tissue homeostasis. The protein level of p53 in NSC34^{G93A} cells showed a significant increase compared to non-transfected cells over three weeks, with fold changes of 3.28, 3.18, and 2.71, respectively (Fig.2.2). Additionally, there was a significant difference in p53 protein levels between the NSC34^{G93A} and NSC34^{WT} groups of cells in both the 1st and 2nd weeks.

(a)



(b)

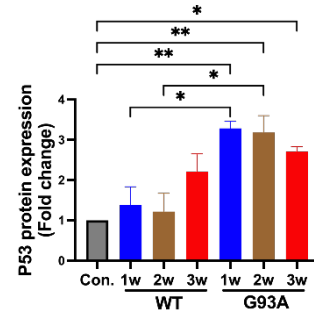


Fig.2.2 The level of p53 protein increases in NSC34^{G93A} cells

(a) Representative Western blot images of p53 protein expressions in non-transfected (control), NSC34^{WT}(green), and NSC34^{G93A} (green) cells in 3 weeks. (b) Quantification of Western blot analysis of p53 protein level in NSC34 cells (n = 5, mean $\pm$ SEM, * p <0.05, ** p <0.01).

After being cultured for three weeks, NSC34^{G93A} cells demonstrated an increased cell size accompanied by numerous small vacuoles in the cytoplasm compared to NSC34^{WT} cells indicating highly activated lysosomes. We applied a SA- β -gal assay to assess cellular senescence further. Based on the data presented in Figure 2.3, it is evident that NSC34^{G93A} cells exhibited significantly higher lysosomal activity compared to NSC34^{WT} cells in the 3rd week. The analysis revealed that 76.69% of SA- β -gal-positive cells were observed in NSC34^{G93A} cells, while only 17.35% were in NSC34^{WT} cells. This finding strongly supports the notion of increased lysosomal activity in NSC34^{G93A} cells.

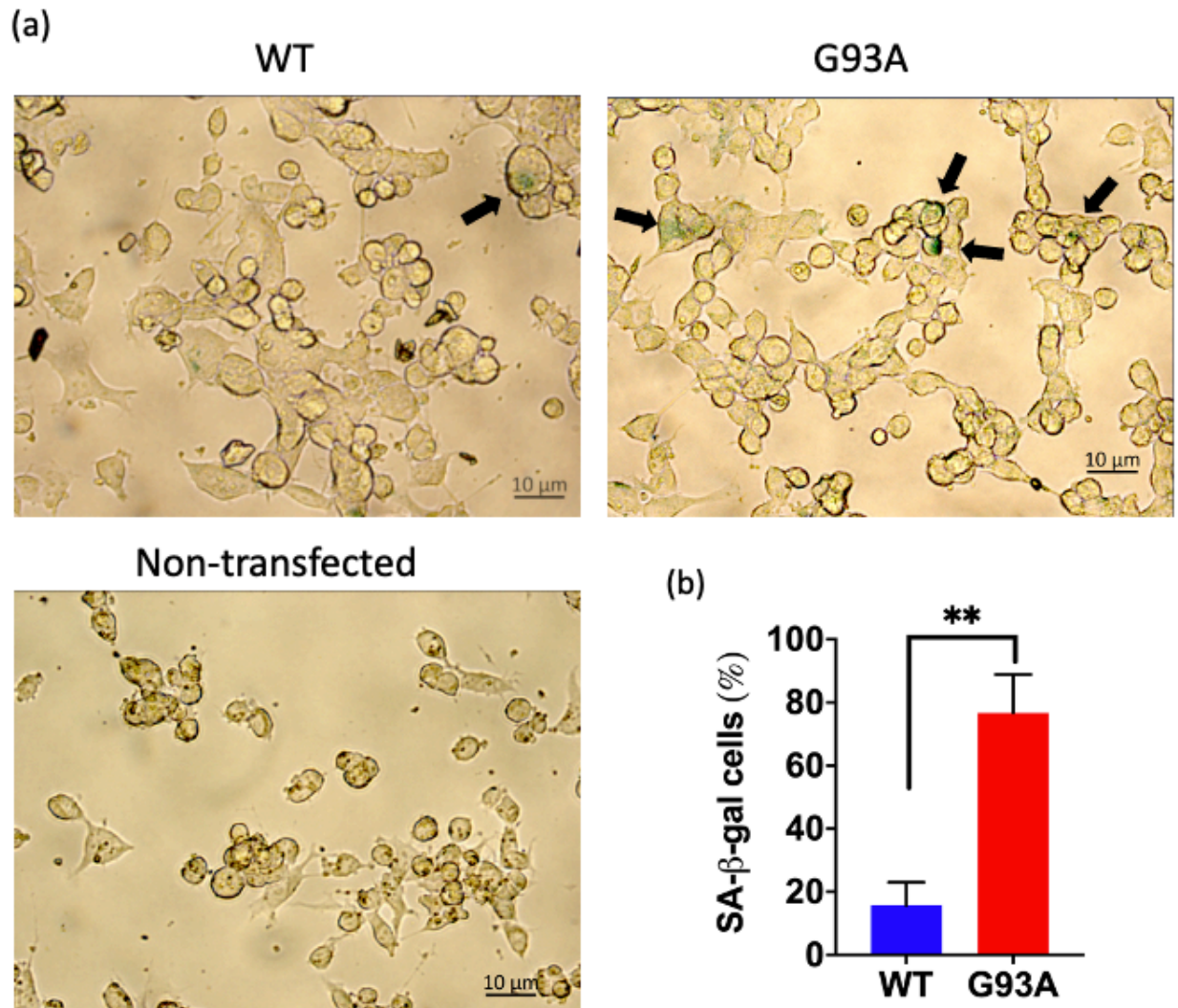


Fig.2.3 NSC34^{G93A} cells show high level of β-gal-positive cells

(a) Representative images of cellular senescence associated with β-galactosidase (SA-β-gal) staining in NSC34 cells. The black arrow indicated the SA-β-gal positive cells (green). Scale bar, 10μm. **(b)** The proportion of SA-b-gal positive cells in total cells from each image field (n=50-100 cells, mean ± SEM, ** $p<0.01$).

Senescent cells exhibit the release of proinflammatory damage-associated molecular patterns, notably high mobility group box 1 (HMGB1), from the nucleus to the cytosol. Protein lysate

derived from NSC34 cells was subjected to centrifugation to separate the nucleus and cytoplasm fractions, as validated by the presence of specific purification markers (Histone H1 for nucleus and β -actin for cytoplasmic fraction). The translocation of HMGB1 from the nucleus to the cytoplasm in NSC34^{G93A} cells was confirmed through immunofluorescence staining and Western blot analysis (Fig. 2.4). To assess the level of hSOD1 oxidation, cell lysates were applied to a plate to immobilize hSOD1. An ELISA assay was performed to quantify the amount of reactive thiol group in hSOD1 (Fig. 2.5). There was a (76.77 ± 6.056) % reduction in the ratio of the thiol group of hSOD1 in NSC34^{G93A} cells compared with that in hSOD1^{WT} cells indicating a higher level of oxidative hSOD1. These findings demonstrate that NSC34^{G93A} cells with higher amounts of oxidized hSOD1 undergo early entrance into cellular senescence.

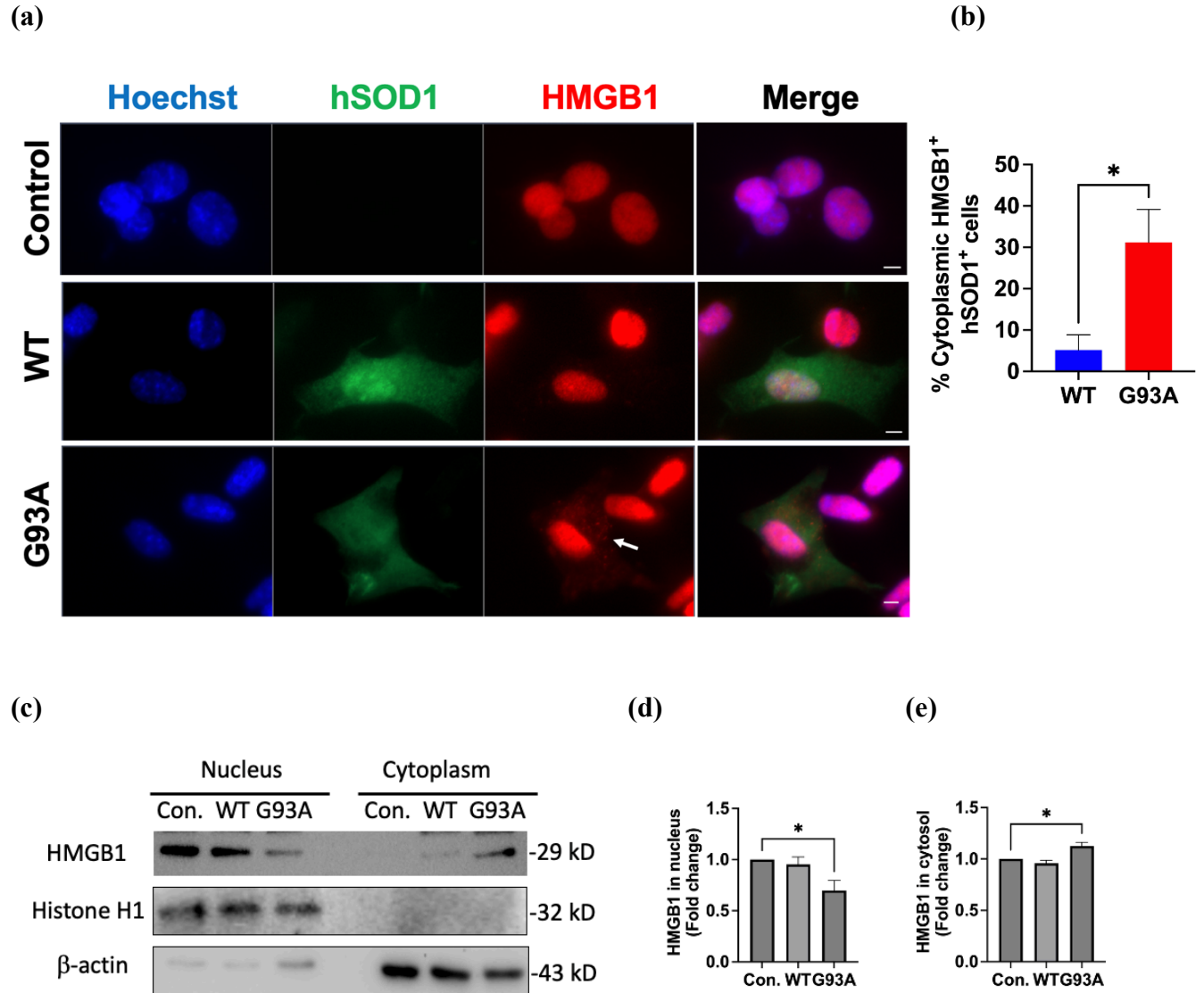


Fig.2.4 Cytoplasmic translocation of HMGB1 is shown in NSC34^{G93A} cells

(a) Immunofluorescence staining for hSOD1 (green), HMGB1 (red), and Hoechst (blue) in NSC34 cells. Scale bar, 10µm. **(b)** The proportion of cells with cytoplasmic translocation of HMGB1 in the total hSOD1⁺ cells (n=20 cells, mean ± SEM, **p* < 0.05, ***p* < 0.01). **(c-e)** Representative and quantification of Western blot analysis of HMGB1 protein levels in the nucleus and cytosol of NSC34 cells (n = 5, mean ± SEM, **p* < 0.05).

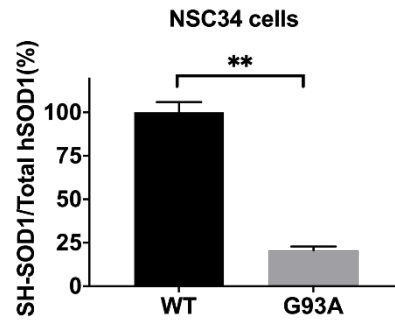


Fig. 2.5 Thiol group of hSOD1 reduces in NSC34^{G93A} cells The proportion of thiol group level in total hSOD1 within NSC34 cells (n=11, mean $\pm$ SEM, ** $p < 0.01$).

2.3.2 Senescent neural stem cells expression hSOD1 contain oxidized hSOD1

Then we did further experiments on neural stem cells (NSCs) to investigate how hSOD1 induces cellular senescence. Previously we reported that oxidation of hSOD1 occurred at its cysteine 111 residue^{414,415}. Once oxidized, hSOD1 undergoes aberrant misfolding. We first determined the expression of misfolded hSOD1 in NSCs after repeated passages, a model of NSC senescence. NSCs were prepared from transgenic mice expressing the full length of human wild-type *SOD1* (NSCs^{WT}). The cells were maintained in Complete StemPro NSC media to form neurospheres and then subcultured at a 1:3 ratio every 2-3 days. Cells from passage 1 (P1), passage 3 (P3) and passage 5 (P5) were used for experiments. NSCs were immunological with antibodies to Ki67 and SOX2 at P5, which was around 2 weeks of culture *in vitro* (Fig. 2.6a and b). About 58% of passage-matched non-transgenic NSCs (NSCs^{-/-}) were positive for Ki67 at P5. Only 30% of cells were positive for Ki67 in the NSCs^{WT}, a significant drop of 51.8% compared to the NSCs^{-/-}. We further analyzed the expression of the oncoprotein p53, a marker of cellular senescence, in whole-cell lysates of NSCs^{WT} from passages 1, 3 and 5. While NSCs^{-/-} didn't express p53 throughout the 5

passages (data not shown), NSCs^{WT} and NSCs^{G93A} showed upregulation of p53 after each passage. Specifically, in the NSCs^{WT} group, p53 increased 4.3-fold in P3 and 7.3-fold in P5 compared to P1. In the NSCs^{G93A}, levels of p53 increased by 6.8-fold in P3 and nearly 12-fold in P5 compared to P1 (Fig. 2.6 c-d).

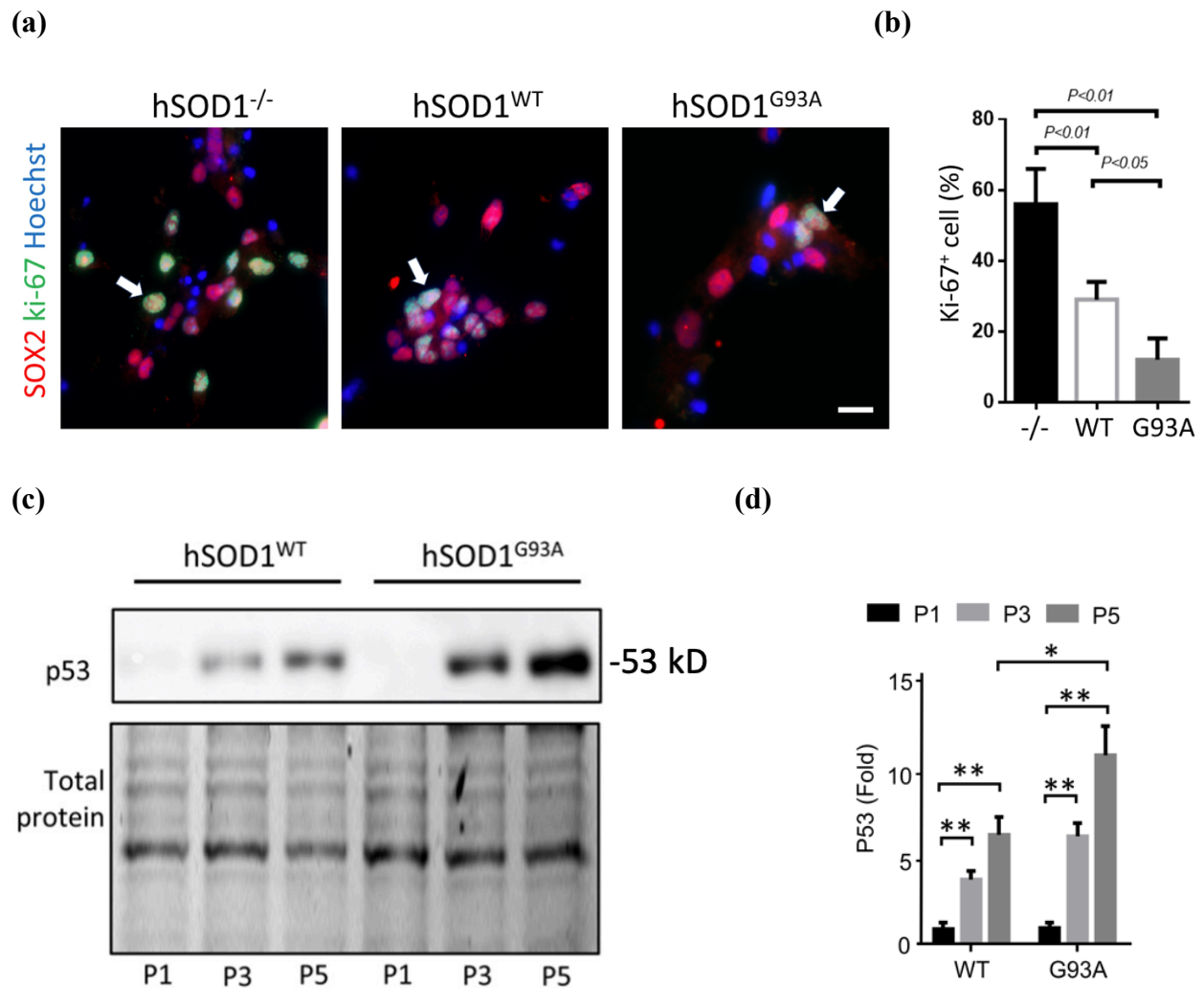
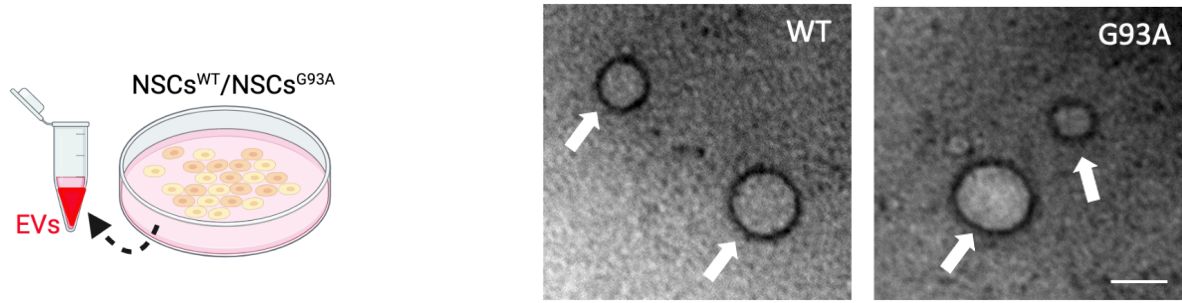


Fig.2.6 NSCs undergo early entrance into replicative senescence in the presence of misfolded hSOD1 (a-b) Expression of the WT hSOD1 and the hSOD1 G93A mutation in NSCs results in diminished proliferation. Cell proliferation was visualized by immunostaining with Ki67 (Green) and neural stem cell marker SOX2 (Red) in P5 NSCs^{WT}. The colocalization of Ki67 with SOX2

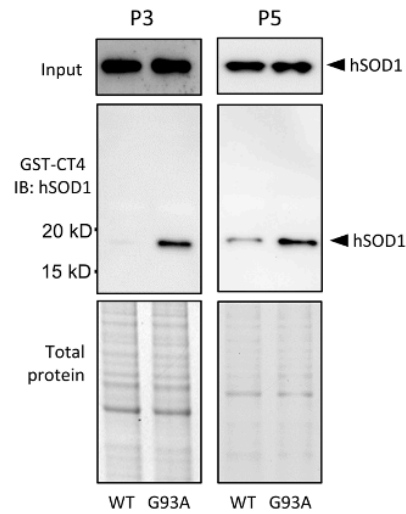
was indicated by the white arrow. Scale bar: 20 μ m. One-way ANOVA followed by Bonferroni's Post-Test. **(c-d)** Western blot detection of p53 at different passages of NSCs^{WT} and NSCs^{G93A}. Two-way ANOVA followed by Dunn-Sidak's multiple comparisons test was performed. All analysis was done in triplicate from three independent cultures.

Pathogenic hSOD1 is known to propagate through protein secretion and EVs⁴¹⁶. To determine if senescent NSCs secrete misfolded hSOD1, we prepared EVs from culture media of mouse NSCs carrying *hSOD1*^{WT} (EVs^{WT}) or *hSOD1*^{G93A} (EVs^{G93A}) at passages 1, 3 and 5, respectively. Electron microscopy of whole-mount EVs revealed a diameter ranging between 50-150 nm but without significant morphological difference between EVs^{WT} and EVs^{G93A} at P5 (Fig. 2.7a). Using a GST-CT4 pulldown assay, a band of misfolded hSOD1 was detected in NSCs^{WT} at P3. Levels of misfolded hSOD1 in NSCs^{WT} increased significantly at P5 (Fig. 2.7b). At passage 1, there was no detectable misfolded hSOD1 in the NSCs^{WT} (Data not shown). Using non-reducing SDS-PAGE (-2-Mercaptoethanol/ +iodoacetamide), we further confirmed the presence of disulfide cross-linked SOD1 of high molecular weight accumulated in both the EVs^{WT} and EVs^{G93A} (Fig. 2.7c). To test if misfolded hSOD1 in EVs was oxidized, we aliquoted the EVs lysates and incubated them in an aerobic or anaerobic chamber overnight. The lysates were then added to a plate to immobilize hSOD1. ELISA was then used to determine the Δ -SH. As shown in Fig. 2.7d, there was an apparent reduction in Δ -SH from P1 ($29.7 \pm 6.5\%$) to P3 ($17.2 \pm 8\%$) and P5 ($12.08 \pm 5.8\%$) in EVs^{WT}, and the reduction was more pronounced in EVs^{G93A}. The findings suggest that cellular senescence induced by hSOD1, whether it is the *hSOD1*^{G93A} mutation or the *hSOD1*^{WT}, may be further exacerbated by EVs containing oxidized hSOD1.

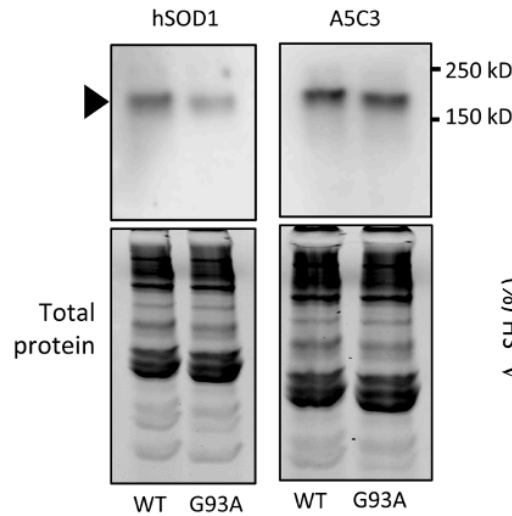
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(b)



(c)



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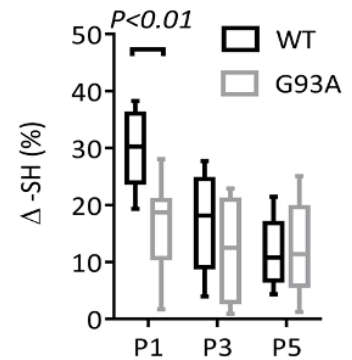


Fig.2.7 Extracellular vesicles from senescent NSCs contain oxidized hSOD1

(a) The EVs, indicated by the white arrows, were observed using EM and exhibited a diameter ranging between 50-150 nm. Scale bar: 100nm. (b) Level of misfolded hSOD1 in EVs isolated from P3 and P5 NSCs^{WT} and NSCs^{G93A} as detected by affinity binding assay with the GST-tagged CT4. Blots were probed with an anti-hSOD1 antibody. Input: total SOD1 in each lysate. (c) SOD1 expression in EVs from P5 NSCs^{WT} and NSCs^{G93A} was analyzed by non-reducing (β -mercaptoethanol and iodoacetamide) SDS-PAGE with antibodies to hSOD1 and misfolded hSOD1 (A5C3). The experiments were done in triplicate from three independent cultures.

(d) Thiol-disulfide status of the SOD1 in EVs^{WT} and EVs^{G93A} from indicated passages of NSCs. The thiol content was normalized to total hSOD1. Two-way ANOVA followed by Dunn-Sidak's multiple comparisons test was performed.

2.3.3 EVs from senescent NSCs induce premature senescence in young NSCs

Our data above demonstrate that senescent hSOD1-carrying NSCs secrete EVs that contain a considerable amount of oxidized SOD1. To further test the effect of EVs from senescent NSCs on young NSCs, EVs^{WT} and EVs^{G93A} at P5 were first labelled with Texas Red and then proportionally applied to P3 NSCs^{WT} for 4 hours. Internalization of EVs was detected in the cytoplasm of NSCs^{WT} (Fig.2.8).

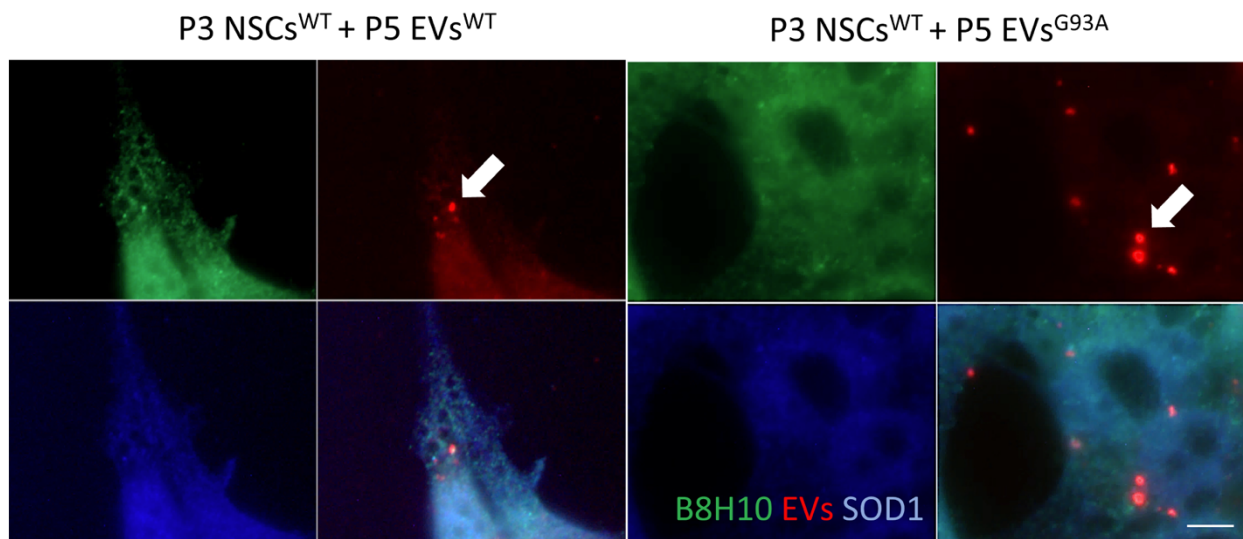
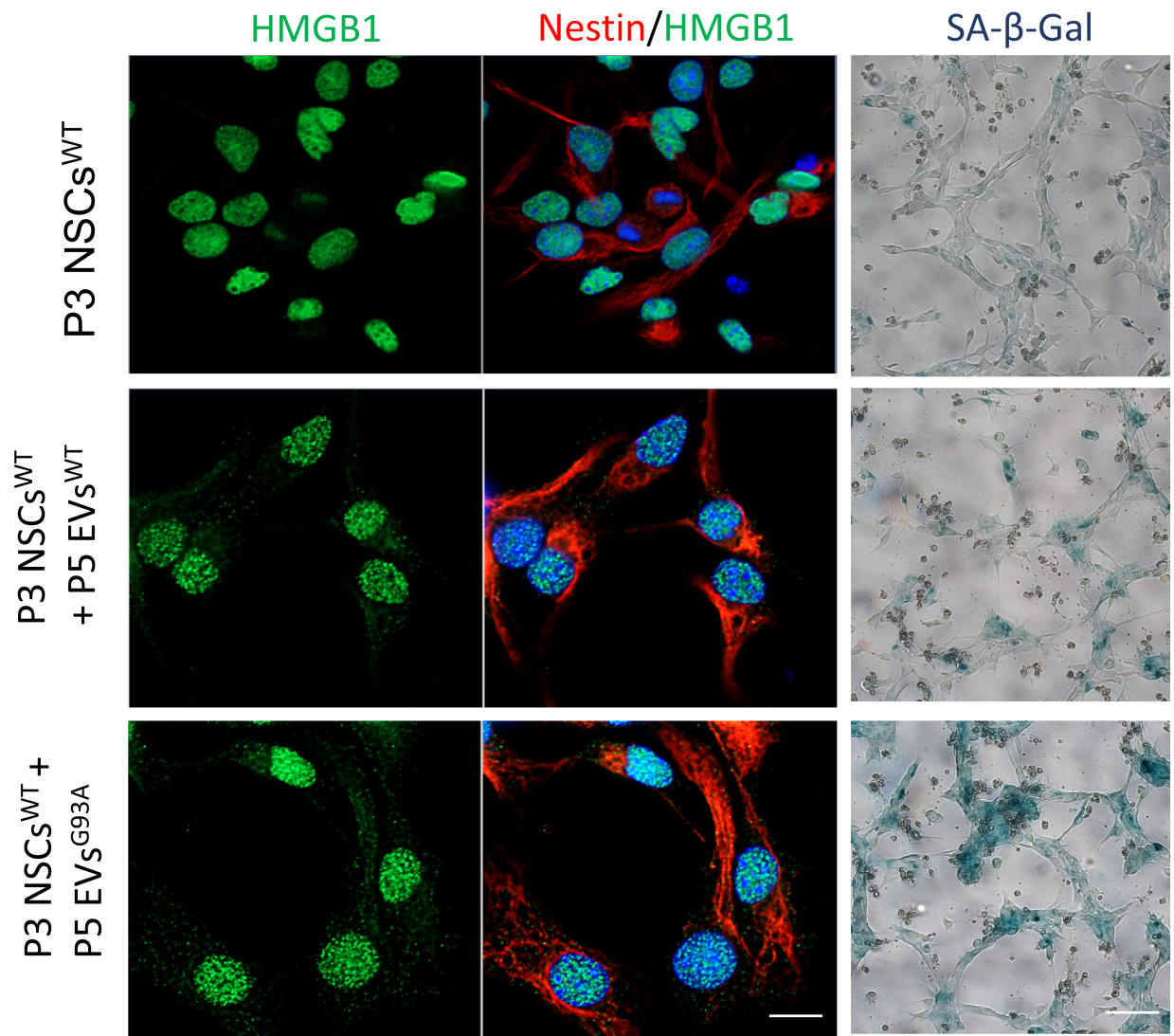


Fig.2.8 Internalization of exogenous EVs from senescent NSCs

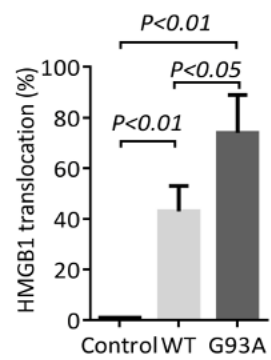
Internalization of exogenous EVs. Representative immunofluorescence staining of misfolded hSOD1 (B8H10, Green) and hSOD1 (blue) after treatment with EVs^{WT} and EVs^{G93A} from P5 of NSCs^{WT}. EVs were labelled with Texas Red and were indicated by the white arrows. Scale bar: 2 μ m.

At 24 hours, the labelled EVs^{WT} were co-localized with misfolded hSOD1 that was immunologically detected with the B8H10 antibody (Fig.2.9a). Immunocytochemistry revealed cytoplasmic translocation of HMGB1 in 43% of the P3 recipient NSCs^{WT} treated with P5 EVs^{WT}, whereas in the control group, HMGB1 translocation was found in less than 2% of the cells. The P5 EVs^{G93A} induced an even higher rate (73%) of translocation of HMGB1 in the P3 NSCs^{WT} (Fig.2.9b). Meanwhile, 58% of P3 NSCs^{WT} treated with P5 EVs^{WT} were positive for SA-β-gal as compared to 13% in control and 73% in the P5 EVs^{G93A}-treated group (Fig.2.9c). The expression of p16, a cyclin-dependent kinase inhibitor known to induce G1 cell cycle arrest, was found to be significantly increased by 1.6-fold in the P5 EVs^{WT}-treated cells compared to the non-treated control, while cells treated with P3 EVs^{WT} didn't show a significant difference (Fig.2.9 d and e). Upregulation of the aging marker p53 was 1.7-fold in the P5 EVs^{WT}-treated cells compared to the control (Fig. 2.9 f and g). These results indicate extracellular vesicles with a high amount of oxidized hSOD1 induce premature senescence in young NSCs.

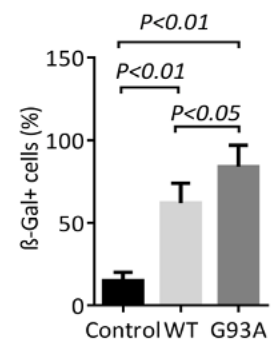
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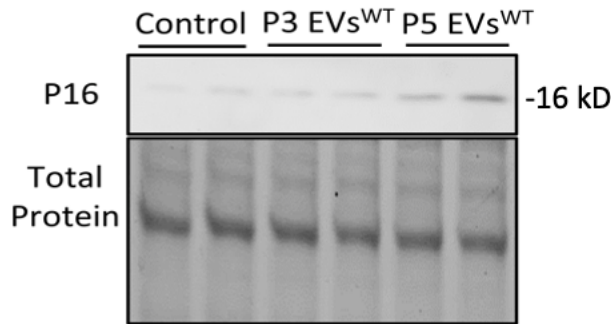
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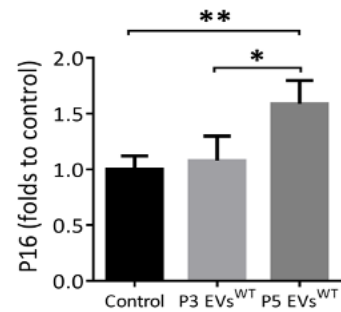
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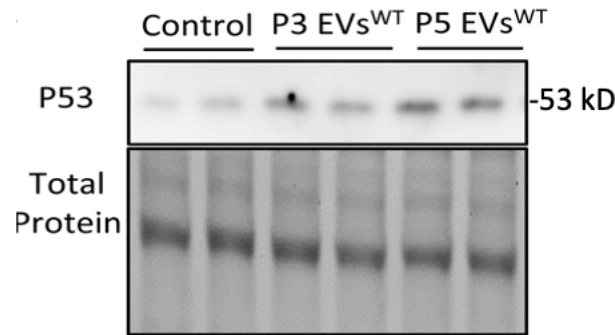
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(e)



(f)



(g)

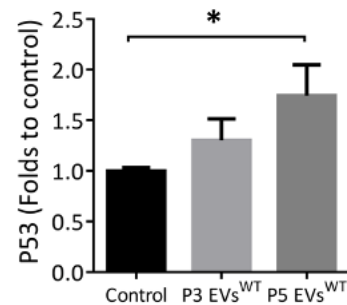
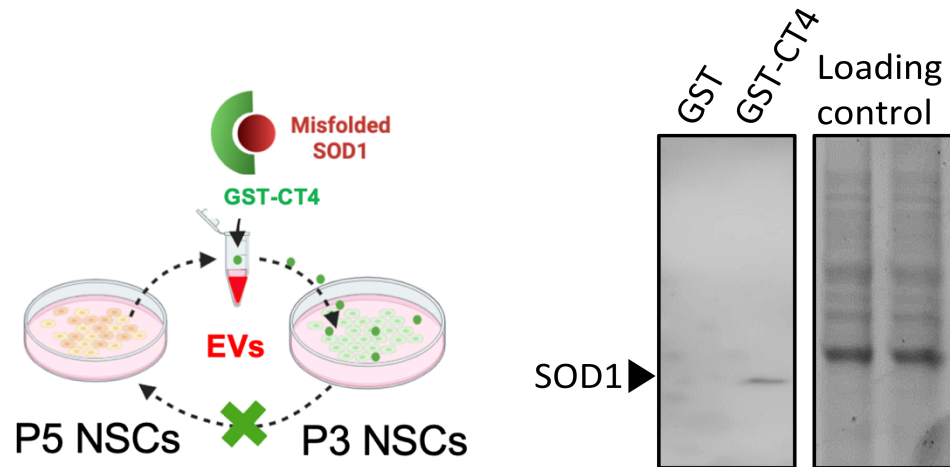


Fig.2.9 EVs from senescent NSC induces aging-like phenotypes (a) Immunofluorescence staining of P3 NSCs^{WT} with HMGB1 (Green) and Nestin (Red) after treatment with P5 EVs^{WT} and EVs^{G93A}. Senescent NSCs were detected by cytoplasmic translocation of HMGB1 and by expression of SA- β -gal. Scale bar: 10 μ m. (b) The translocation of HMGB1 from the nucleus to the cytosol in the P3 recipient NSCs^{WT}. (c) Quantification of SA- β -gal positive cells. For C and D, one-way ANOVA followed by Bonferroni's Post-Test was performed. The experiments were conducted in triplicate from three independent cultures. (d-g) Expression of p16 and p53 detected by Western blot in P3 NSCs^{WT} treated with P3 or P5 EVs^{WT} for 24 h. One-way ANOVA followed by Bonferroni's Post-Test was performed.

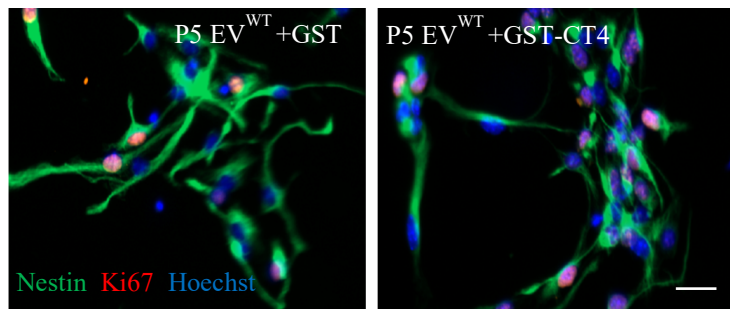
2.3.4 Targeting oxidized hSOD1 mitigates neurotoxicity of EVs from senescence NSCs

To investigate whether oxidized hSOD1 in EVs is the causal factor of aging, we targeted misfolded hSOD1 from the EVs^{WT} by the GST-CT4 affinity capture to test whether this way could restore the proliferation of NSCs. Intact exosomes were incubated with GST-CT4 bound Glutathione Sepharose. The GST-CT4, but not the GST alone, pulled down a SOD1 band from the lysates of P5 EVs^{WT} (Fig. 2.10a). After the affinity capture of misfolded SOD1, the P5 EVs^{WT} was applied to P3 NSCs. The number of Ki67⁺ cells was restored to $28 \pm 7.5\%$ from $17 \pm 5.5\%$ in GST-alone control (Fig. 2.10 b and c).

(a)



(b)



(c)

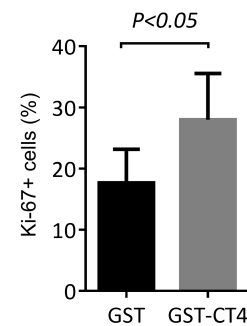


Fig.2.10 Removal of misfolded SOD1 from P5 EVs^{WT} restored proliferation of NSCs (a)

Incubation of GST-tagged CT4 and P5 EVs^{WT} for 2 h resulted in a pulldown of a misfolded SOD1 band as detected by Western blot. **(b)** After the removal of misfolded SOD1, P5 EVs^{WT} lost its inhibition on the proliferation of P3 NSCs^{WT} as determined by immunostaining with antibodies to Ki67 (Red) and Nestin (Green). Scale bar: 20 μ m. **(c)** For quantitative comparison of Ki67 positive cells, a Student's t-test was performed. The experiments were done in triplicate from three independent cultures.

We conducted further experiments to explore the toxicity of P5 EVs in neuronal cultures. The isolated P5 EVs^{WT} were incubated for 2 h at 37°C with the CT4 motif (FLYRWLPSRRGG) to block oxidized SOD1 before they were applied to P3 NSCs^{WT}. When oxidized SOD1 was blocked with CT4, the P5 EVs^{WT} induced a 53% decrease of total hSOD1 in the detergent-insoluble fraction of the P3 NSCs^{WT}, while in the soluble fraction, it was not significantly decreased after 24 h (Fig. 2.11 a and b). Next, we exposed the P3 NSCs^{WT} in differentiation medium to P5 EVs^{WT} that were treated with/without CT4 for 24 hours. Immunocytochemistry confirmed that the application of P5 EVs^{WT} induced the formation of SOD1 aggregates (Fig. 2.11c). Application of P5 EVs^{WT} to the recipient P3 NSCs^{WT} induced a significant loss of MAP2-positive neurons and an increased release of lactate dehydrogenase (LDH), which was similar to the toxicity induced by P5 EVs^{G93A}. Pre-incubation with CT4 increased MAP2-positive neurons by approximately 50% (Fig. 2.11d) and a significant decrease in the release of lactate dehydrogenase induced by P5 EVs^{WT} (Fig. 2.11e). Therefore, targeting oxidized hSOD1 mitigates the neurotoxicity of EVs, indicating the crucial role of oxidized hSOD1 in aging.

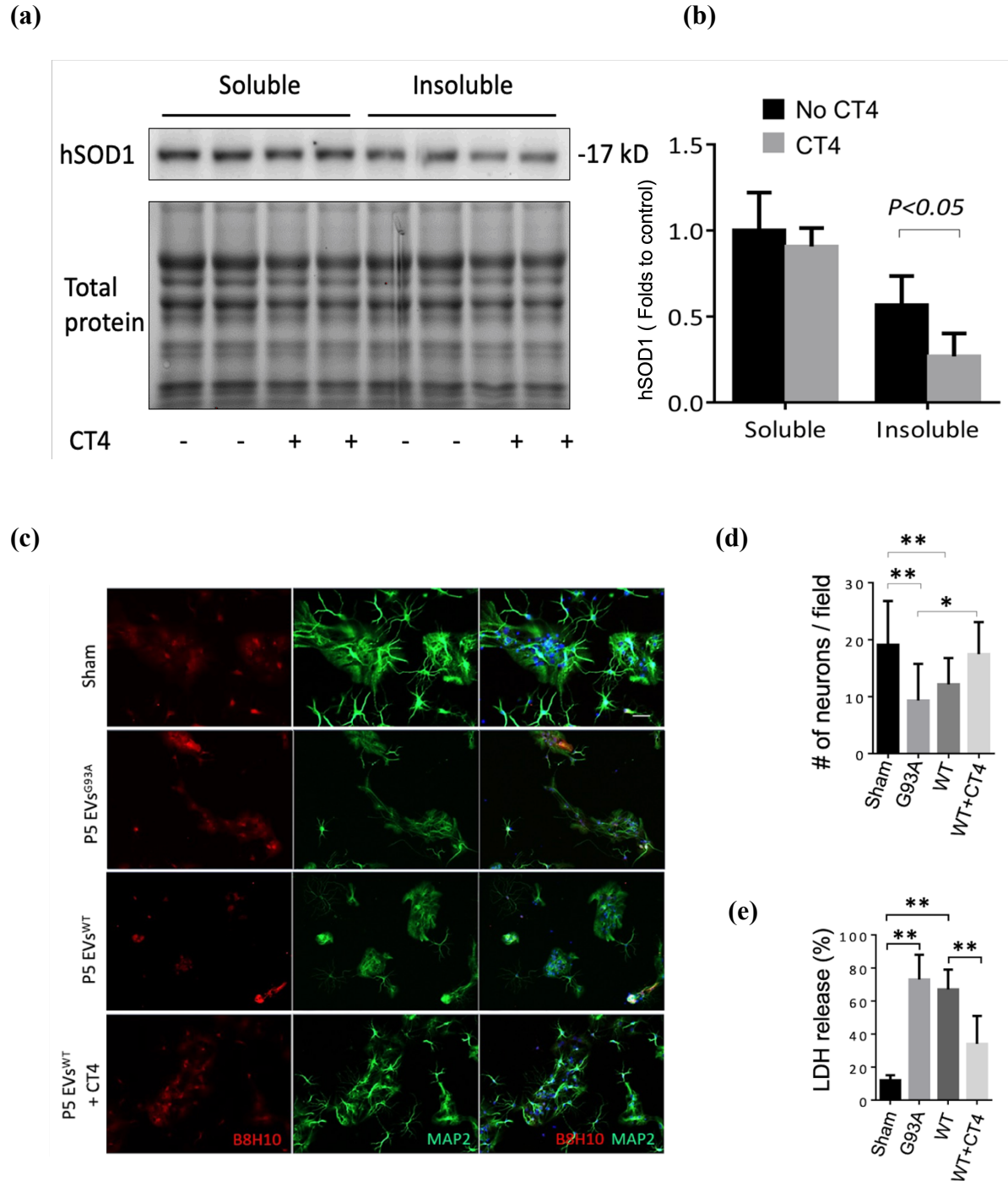


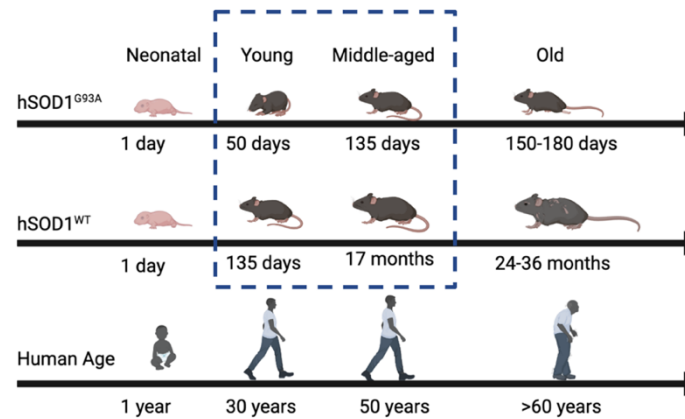
Fig.2.11 Blocking misfolded SOD1 reduces SOD1 aggregation and mitigates neurotoxicity of EVs isolated from senescent NSCs (a-b) P5 EVs^{WT} was incubated for 2 h with or without the CT4 motif (FLYRWLPSRRGG) before it was added to P3 NSCs^{WT}. After 24 h, protein samples were separated into detergent-insoluble and soluble fractions and analyzed by Western blot with

an anti-hSOD1 antibody. Total protein generated by stain-free visualization was used to ensure equal loading. Two-way ANOVA followed by Dunn-Sidak's multiple comparisons test was performed. **(c)** Neurotoxicity of P5 EVs to P3 NSCs^{WT}. Cells were immunostained with antibodies to misfolded hSOD1 (B8H10, Red) and MAP-2 (Green). Scale bar: 50µm. **(d)** Quantification of MAP-2 positive neurons. **(e)** Release of lactate dehydrogenase. For D and E, one-way ANOVA followed by Bonferroni's Post-Test was performed. The experiments were done in triplicate from three independent cultures.

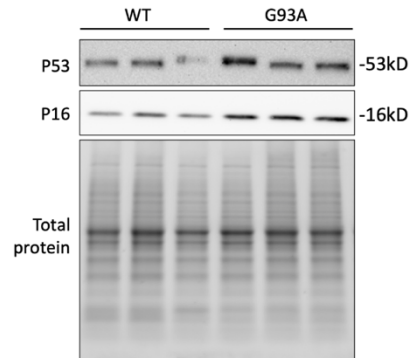
2.3.5 Oxidized hSOD1 is a causal factor of aging in hSOD1 transgenic mice

To further confirm the role of oxidized hSOD1 during aging process, we conducted an *in vivo* study using transgenic mice expressing *hSOD1*^{G93A} and *hSOD1*^{WT}. Mice were categorized into different age groups for further experiments (Fig.2.12a). The average lifespan of hSOD1^{G93A} mice monitored in our lab was 165±15 days. We classified hSOD1^{G93A} mice into three different age groups: 50 days (young), 135 days (middle-aged), and above 150 days (old). On the other hand, hSOD1^{WT} mice exhibited a significantly longer lifespan of 29.8 ± 2.1 months⁴¹⁷. To compare with hSOD1^{G93A} mice, we classified hSOD1^{WT} mice into three different age groups: 135 days (young), 17 months (middle-aged), and 30 months (old). To further validate the progression of aging in hSOD1 transgenic mice, we assessed the levels of aging biomarkers p53 and p16, which were previously demonstrated *in vitro*. The analysis of brain tissue revealed that the protein level of p53 was 2.21-fold higher, and p16 was 1.45-fold higher in hSOD1^{G93A} mice compared to hSOD1^{WT} mice (Fig. 2.12 b and c). Furthermore, in liver tissue, the protein expression of p53 and p16 was 2.07-fold higher and 1.69-fold higher in hSOD1^{G93A} mice compared to hSOD1^{WT} mice (Fig. 2.12 d and e).

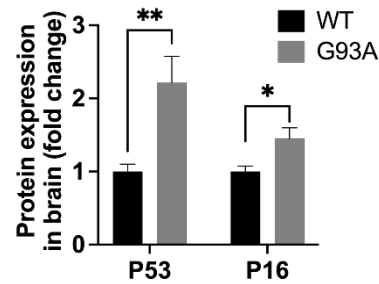
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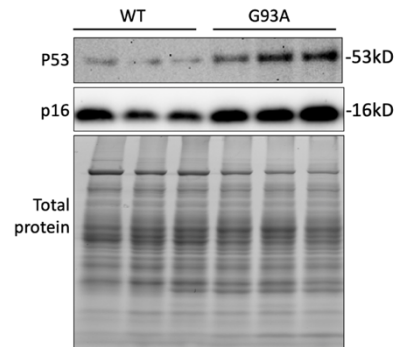
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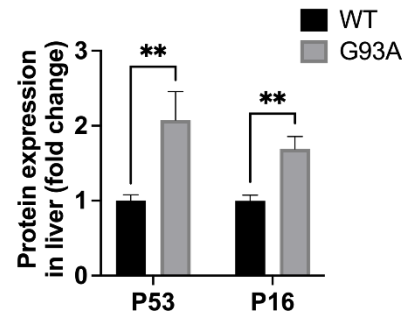


Fig.2.12 Aging biomarkers increase in both brain and liver tissue of hSOD1^{G93A} transgenic mice (a) Transgenic mice expressing *hSOD1^{G93A}* and *hSOD1^{WT}* were divided into different age groups. (b) The levels of aging biomarkers, including p53 and p16 were analyzed by Western blot

in the brain tissues **(b-c)** and liver tissues **(d-e)**. Each experiment was repeated independently 3 times. Data are expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

To investigate oxidative stress *in vivo*, we examined various indicators of oxidation in transgenic mice expressing *hSOD1^{G93A}* and *hSOD1^{WT}* at 135 days of age. The data revealed that protein carbonyl levels were significantly elevated, showing a 1.48-fold increase in *hSOD1^{G93A}* mice compared to *hSOD1^{WT}* mice (Fig. 2.13a). Furthermore, we assessed malondialdehyde (MDA), a prominent product resulting from lipid peroxidation within cells. Our findings demonstrated that MDA levels in the brain were 1.53-fold higher in *hSOD1^{G93A}* mice compared to those in *hSOD1^{WT}* mice. (Fig.2.13b). Furthermore, we tested the percentage of the thiol group of hSOD1 in total hSOD1 by Elisa test in *hSOD1^{WT}* and *hSOD1^{G93A}* mice. The results showed a lower ratio of thiol-/total hSOD1, possibly due to increased oxidation of hSOD1(Fig.2.13c). Consistent with our previous studies, when we selectively knock down the oxidized hSOD1 by CT4 peptide, it could also lead to an upregulation of the thiol group of hSOD1 (Fig. 3.13d). These findings provide additional evidence *in vivo* that oxidized hSOD1 is one of the contributing factors to the aging process.

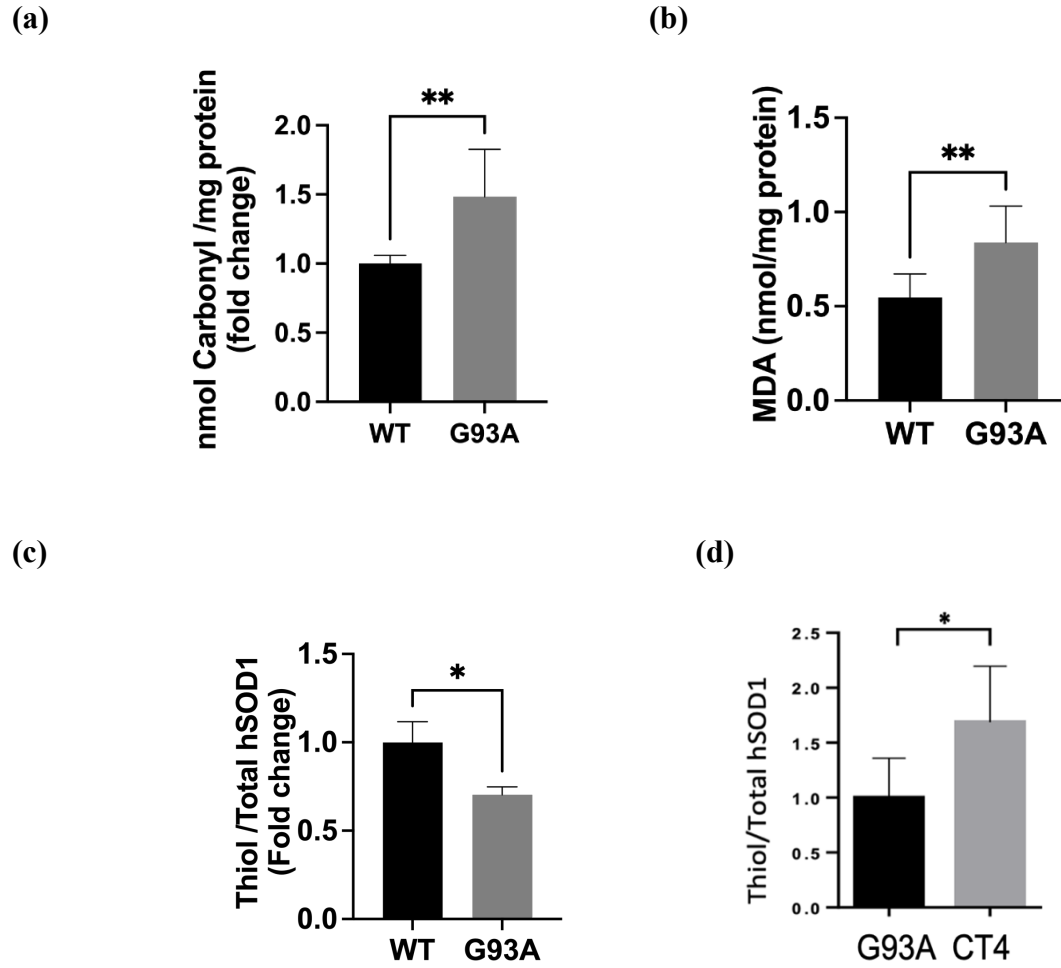


Fig. 2.13 Oxidative modifications in the brain of hSOD1 transgenic mice (a-b) The levels of carboxyl and MDA were quantified in the brains of 135-day-old hSOD1WT and hSOD1G93A transgenic mice (n=6). (c) The thiol group level in total hSOD1 was quantified in the brains of 135-day-old hSOD1WT and hSOD1G93A transgenic mice (n=6). (d) The levels of oxidized hSOD1 were quantified in the brains of hSOD1G93A mice at 50, 100, 120, and 150 days (n=3). Each experiment was repeated independently 3 times. Data are expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

2.4 Discussion

Aging is commonly accompanied by a decrease in enzyme levels or activity, and in some cases, proteins that have undergone post-translational modifications acquire toxic functions, leading to an accumulation of oxidants and an acceleration of the aging process ⁴¹⁸. While there is evidence of oxidant accumulation in age-related diseases, the exact causal factor of aging remains elusive.

Cellular senescence, one of the hallmarks of aging, refers to persistent cell cycle arrest. It can be triggered in normal cells by various internal and external stimuli and is believed to be a complex and dynamic process. A growing body of evidence suggests that reactive oxygen species (ROS) play a significant role in cellular senescence ⁴¹⁹. Although compelling correlational data supports the oxidative stress theory, a direct cause-and-effect relationship between the accumulation of oxidative damage and cellular senescence has yet to be definitively established ^{420,421}.

Mouse motor neuron NSC34 cells are a hybrid cell line produced by the fusion of motor neuron enriched, embryonic mouse spinal cord cells with mouse neuroblastoma. Motor neurons are vulnerable to cellular senescence ⁴²². Firstly, motor neurons have an extended lifespan compared to other cell types, which increases the likelihood of accumulating age-related changes and experiencing cellular senescence. Additionally, motor neurons exhibit high metabolic activity and energy requirements, generating reactive oxygen species (ROS) as by-products. The resulting oxidative stress can damage cellular components and contribute to senescence. Moreover, motor neurons have limited regenerative capacity, impeding their ability to replace damaged or lost cells efficiently. This limitation renders them more susceptible to senescence and functional decline. Furthermore, motor neurons are prone to protein misfolding and aggregation, particularly in neurodegenerative diseases like amyotrophic lateral sclerosis (ALS) ²⁸⁹. The accumulation of misfolded proteins activates stress response pathways, ultimately leading to cellular senescence.

Lastly, motor neurons are constantly exposed to excitotoxicity, excessive stimulation of excitatory neurotransmitters such as glutamate, which can cause calcium influx and subsequent cellular damage ⁴²³. This excitotoxicity further promotes senescence and contributes to neuronal dysfunction. Understanding these vulnerabilities is crucial for elucidating the mechanisms underlying motor neuron senescence and developing strategies to mitigate age-related neurodegenerative conditions while preserving neuronal health during aging.

Senescence encompasses numerous cellular and molecular alterations, along with diverse phenotypic changes. However, none of these factors alone is the definitive causal factor of aging. Extensive techniques have been employed to demonstrate phenotypes of cellular senescence. Our study showed that the hSOD1^{G93A} mutation induces permanent cell cycle arrest in NSC34 cells by using an EdU assay. p16, also known as cyclin-dependent kinase inhibitor 2A (CDKN2A), is a tumor suppressor protein that functions as a negative cell cycle regulator. It inhibits the activity of CDKs involved in cell cycle progression ⁴²⁴. Activation of p16 can occur in response to various stimuli, including oncogenic signalling, DNA damage, and telomere dysfunction ⁴²⁵. Although these senescent cells cease cell division, they maintain viability and undergo significant changes in metabolic activity and gene expression, developing a complex SASP ⁹. The SASP involves the secretion of various factors, including lysosomal enzymes, pro-inflammatory cytokines and HMGB1. In individuals with Alzheimer's disease and aged rodents, an elevated release of HMGB1 in the brain and cerebrospinal fluid indicates impaired gene repair mechanisms ⁴²⁶. *P53* is known as the "guardian of the gene" because it maintains genomic stability and prevents the proliferation of damaged or potentially cancerous cells. It is often activated in senescent cells as a response to DNA damage or other stress signals. Once activated, *P53* can regulate numerous downstream processes that contribute to senescence ³². Our findings provide evidence that hSOD1^{G93A} NSC34

cells exhibit a group of senescent characteristics, such as increased cell size, the elevated protein level of p53, enhanced lysosome activity (as indicated by SA- β -gal positive cells) and translocation of HMGB1. In conclusion, our study provides compelling evidence that the hSOD1^{G93A} mutation initiates early senescence in NSC34 cells. Moreover, we have further confirmed that this effect extends to neural stem cells (NSCs), as hSOD1^{G93A} can induce NSCs to premature senescence after repeated passages. Our findings also highlight the intriguing observation that mouse NSCs expressing the wild type of hSOD1 exhibit the expression of cellular senescence biomarkers as early as passage 5. Based on these findings, we assume that hSOD1 may play a role in promoting cellular senescence that needs to be further investigated.

Aging leads to disrupted cell metabolism and results in the intracellular accumulation of misfolded and aggregated proteins ⁴²⁷. Protein misfolding is both a cause and a consequence of increased cellular stress and has been observed in the nervous tissues of patients affected by a variety of age-related disorders, such as Alzheimer's disease and ALS ⁴²⁸. The ability of NSCs to self-renew and differentiate into multiple neural lineages makes them indispensable in maintaining a multipotent reservoir and generating differentiated cells. A recent study shows that self-association and subsequent deposition of the misfolded protein aggregates are primarily present in the resting NSCs, and the accumulation of protein aggregates that are contained within lysosomes in healthy young adult NSCs play a physiological role since they are required for NSCs mobilization and activation ⁴²⁹. Young stem cells are relatively robust to handling misfolded protein through high levels of the ATP-dependent TRiC/CCT Chaperonin ³⁹⁶. In aged NSCs, the ability to reactivate (i.e., re-entry to the cell cycle) is limited due to fewer lysosomes being available to fuse with autophagosomes for autophagy to remove toxic proteins ⁴²⁹. Defects of lysosomes and accumulation of protein aggregates also display delayed proteotoxicity to differentiated neurons

that are derived from aged NSCs. Manipulation of autophagy to enhance the clearance of protein aggregates has been shown to rejuvenate aged NSCs ⁴³⁰. Given that NSCs can be isolated from the brains of presymptomatic mice but not symptomatic mice carrying the SOD1 G93A mutation, which are known to have aggregate-prone hSOD1 ⁴³¹, we would speculate that oxidized SOD1 contributes to the progression of aging by impairing NSCs proteostasis.

ALS-linked hSOD1 variants have been shown to induce a prion-like transmission ⁴³². Misfolded wild-type hSOD1 exhibits significant toxicity toward cells and is detected in the cerebrospinal fluid (CSF) from patients with sporadic ALS ⁴³³. Thiol oxidation in SOD1 facilitates its misfolding and plays a role in its gain of toxic functions. Cys6, Cys57, Cys111, and Cys146 are four cysteine residues of human SOD1. An internal disulfide bond exists between Cys57 and Cys146, which contributes to the high stability of the SOD1 protein, while the cysteine residues at positions 6 and 111 are free and can be reversibly oxidized to a sulfenic acid (Cys-SOH) by low concentrations of hydrogen peroxide or irreversibly to sulfinic (Cys-SO₂H) / sulfonic (Cys-SO₃H) acids by high concentrations of hydrogen peroxide ⁴³⁴.

Our data show that an elevated level of oxidized hSOD1 in mutant hSOD1^{G93A} transfected NSC34 cells, and oxidation of hSOD1 occurs in NSCs after repeated passages in culture. Oxidized SOD1 inevitably undergoes aberrant misfolding ⁴¹⁴ and is not readily degraded through common protein degradation pathways. As a result, accumulation of oxidized and misfolded hSOD1 is detected in aging and age-related neurodegenerative diseases ^{328,345}. The high susceptibility of hSOD1 to oxidization may provide an explanation for the fact that it is more challenging to grow primary human cells than animal cells in culture. Based on our data, it is tempting to speculate that oxidized SOD1 plays a role in cellular senescence and further research is needed to be fully understood.

Extracellular vesicles serve as a significant element in communicating and coordinating the function of adult neurogenic niches ⁴³⁵. Protein aggregates can be released via exocytosis in the form of extracellular vesicles for toxic protein disposal, which is a hallmark for cells with low lysosome number or lysosomal activity ⁴³⁶. Exosome-based therapies are an emerging field in medicine and hold the potential to revolutionize the treatment of various diseases ⁴³⁷.

To investigate whether there is a causal relationship between oxidized hSOD1 and cellular senescence, we collected EVs from culture media of senescent NSCs carrying the wild-type *hSOD1* gene containing high levels of oxidized hSOD1. Our data show that EVs from aged NSCs carrying the *hSOD1* gene have considerable levels of oxidized SOD1 and are senescence-inducing and toxic to NSCs and neurons. It is, therefore, necessary to include the level of oxidized SOD1 in the quality control of EV preparation. A previous study has shown that EVs enter target cells but lack of association with lysosomal-associated membrane protein 1 (LAMP1) ⁴³⁸, implying that low degradation efficiency in aged NSCs is a consequence of not only the declined lysosomal activity but also the decreased binding with lysosomes for quick clearance. To investigate the effects of oxidized hSOD1-enriched EVs, we discovered the exogenous EVs from senescent NSCs^{WT} to young NSCs accelerate the senescence of the young NSCs. Furthermore, the removal of misfolded hSOD1 from the EVs by affinity capture abolishes its senescence-inducing activity and restores the proliferation of young NSCs. These findings prove that oxidized hSOD1 is a causal factor of cellular senescence.

Researchers have identified some specific proteins within the cellular senescence pathway ⁴³⁹. These proteins may play key roles in regulating the senescence process, and by modulating their activity, it may be possible to influence cellular senescence and its associated effects on tissues and organs. A category of therapeutic strategies that specifically target proteins to either alleviate

or modulate the senescent state ⁴⁴⁰. These approaches aim to address age-related diseases and conditions associated with the accumulation of senescent cells, as these cells are implicated in various age-related pathologies. Ablation of oxidized proteins in senescent cells has been found to extend health spans and lower the incidence of age-related diseases in a mouse model ⁴⁴¹. This discovery opened the door for the creation of a new class of medications known as protein targets-senotherapeutics, which may modify the structure of proteins and multiple cell signals in senescent cells and delay the start of age-related diseases. Our research team previously demonstrated that a Derlin-1 (CT4) derived peptide could selectively knock down the misfolded SOD1 ⁴⁰⁵. Derlin-1 is an ER membrane protein and plays a vital role in the retro-translocation of misfolded proteins and induces ER stress. Because SOD1 protein has a Derlin-1 binding region (DBR) that is generally embedded but exposed when SOD1 becomes oxidatively modified, this exposed DBR region in misfolded SOD1 could interact with Derlin-1 protein to induce ER stress and cell death ⁴⁴². A Japanese team first discovered the Derlin-1-derived peptide, termed Derlin-1 (CT4), which could be competent with misfolded SOD1 by interacting with Derlin-1 in ER ⁴⁴³. Based on our previous findings, CT4 peptide could selectively downregulate the level of oxidized SOD1 and prolong the lifespan of G93A mice. All those findings indicate that oxidatively modified SOD1 is a key promotor of aging, and downregulation of oxidatively modified SOD1 could be one of the therapeutic targets for anti-aging strategies.

To further confirm the role of oxidized SOD1 during aging process, we conducted an *in vivo* study using transgenic mice expressing *hSOD1^{G93A}* and *hSOD1^{WT}*. Mice were categorized into different age groups for further experiments (Fig.3.13). The average lifespan of *hSOD1^{G93A}* mice monitored in our lab was 165±15 days. Starting from around 120 days of age, these mice gradually displayed symptoms such as limb tremors, hind limb weakness, gait instability, swaying, and eventually

unilateral or bilateral limb paralysis. Ultimately, both hind limbs became immobile, resulting in a loss of independent feeding ability. The experiment was designed with an endpoint at 135 days, during which the incidence of onset or paralysis rarely occurred, indicating a relatively stable phase considered middle age. Furthermore, hSOD1^{G93A} mice aged 50 days were categorized as young, while mice aged above 150 days were classified as old. In contrast, hSOD1^{WT} mice exhibited a significantly longer lifespan of 29.8 ± 2.1 months⁴¹⁷. To compare with hSOD1^{G93A} mice, we classified hSOD1^{WT} mice into three different age groups: 135 days (young), 17 months (middle-aged), and 30 months (old). Protein oxidation may be caused by both direct oxidations of proteins by ROS and indirect oxidative stress modification of proteins by secondary by-products. Secondary modifications include carbohydrates and lipid peroxidation that have been oxidatively changed and subsequently react with proteins. Our results demonstrated the brain tissue indicated higher carbonyl and lipid peroxidation levels than the same age of WT mice but a lower level of the thiol group of hSOD1. It suggests that the G93A mutant SOD1 at an early age had a reduced amount of reactive thiol group. Selective knockdown of misfolded hSOD1 by the CT4 peptide could upregulate the amount of thiol of hSOD1. These findings are consistent with previous *in vitro* evidence.

The present study provides evidence for oxidized SOD1 as a causal factor *in vitro* and *in vivo*. Developing a proof-of-principle that targets oxidized SOD1 could be an effective way to rejuvenate NSCs and improve the quality of EVs prepared from senescent cells.

Chapter III

Impairment of MDV formation is a mechanism of aging

3.1 Introduction

Aging is a universal biological process characterized by ongoing, progressive changes at the cellular and tissue levels that inevitably lead to functional impairments that directly affect cellular, tissue, and organismal functions. As a result, the age-dependent modification of physiological activities diminishes vitality and raises mortality risk with age. In humans, aging is a significant risk factor for many degenerative disorders, including osteoporosis, neurological disorders, diabetes, and cancer ⁴⁴⁴. Mitochondria are highly structured and active organelles essential for cellular metabolism, signalling, and survival. A variety of mitochondrial quality control mechanisms maintain mitochondrial homeostasis. Mitochondria is a crucial organelle closely related to the health of the body and the functioning of tissues, so mitochondrial dysfunction is regarded as a hallmark of aging ⁴². Besides, Accumulating evidence suggests that mitochondrial quality control (MQC) keeps mitochondrial homeostasis but is also an important contributor to aging and age-related diseases ⁴⁴⁵. Mitophagy is the autophagic process of selectively targeting and removing oxidized proteins or damaged parts of mitochondria. The importance of mitophagy in keeping the mitochondrial network healthy has been highlighted by recent discoveries about the intricate interplay between the circuits that regulate it. Mitophagy is generally considered to play a predominant role in eliminating damaged mitochondria ¹⁹⁵, but imbalanced mitophagy could also trigger aging process⁴⁴⁶.

Like other IMS proteins, the import and retention of SOD1 in the IMS depend on its folding and maturation, which involves the production of essential intramolecular and intermolecular disulfide bonds ⁴⁴⁷. The cysteine residues of SOD1 play a critical role in its localization to the mitochondrial intermembrane space (IMS). The import of SOD1 into the IMS is facilitated by its copper chaperone (CCS), which promotes the maturation and retention of SOD1 in the IMS ⁴⁴⁸.

Researchers have found abnormal localization and aggregation of mutant SOD1 in the IMS contributes to mitochondrial abnormalities observed in familial ALS, potentially playing a crucial role in disease development ³⁰¹. Although there is speculation that the presence of SOD1 in the IMS may play a role in eliminating superoxide generated by the mitochondrial respiratory chain, the precise mechanism behind this function remains incompletely understood.

A recent study discovered that mitochondrial-derived vesicles (MDVs) that are LC3-independent vesicles could step in to fill the gap when classical mitophagy is lost ^{220,449}. MDVs are generated from the inner or outer mitochondrial membrane and contain proteins specific to their respective compartments. Electron microscopy studies have revealed that MDVs derived from the outer membrane are single-membrane vesicles⁴⁵⁰. Conversely, MDVs originating from the inner membrane possess two membranes and exclude outer membrane proteins ⁴⁵¹. Notably, high-resolution confocal microscopy has shown that cells lacking autophagy mechanisms produce an increased quantity of MDVs that specifically target lysosomes ⁴⁰⁷. Except for late endosomes/lysosomes, MDVs can also target peroxisomes ⁴⁵². Peroxisomes are the organelle with the closest connection to mitochondria and share many metabolic activities. Moreover, a minority of MDVs may be directed to the plasma membrane and released as extracellular vesicles ²²⁴. MDV trafficking to late endosomes/lysosomes, peroxisomes, and even extracellular release offers a way for recycling and secretion of mitochondrial material to maintain homeostasis post autophagy suppression. These vesicles have been recognized to play a vital role in the disposal of oxidized proteins in both physiological and pathological conditions²¹⁶. However, the fate of MDVs is still debatable.

In summary, MDVs could help mitochondria keep functional, but mitochondria may lose their ability to generate MDVs if they become dysfunctional. Whether MDVs participate in aging still

lacks enough evidence. In our previous study, we monitored MDVs in Chinese hamster ovary (CHO) cells and oligodendrocyte progenitor cells (OPCs)⁴¹¹. In the present study, we established a method to isolate MDVs from liver mitochondria. We found MDVs containing oxidized hSOD1 generate more frequently in middle-aged mitochondria compared to young ones, suggesting that MDVs may serve a role in eliminating oxidized proteins during the aging process. We also compare the yield of MDVs by incubating mitochondria with antimycin A (AA) and found middle aged mitochondria couldn't handle the oxidative stress, indicating the mitochondrial responsiveness decreases with advancing age. Based on these findings, yield of MDVs and mitochondrial responsiveness provide us with novel indicators of aging.

3.2 Material and Methods

3.2.1 Animal models

The transgenic mouse model was congenic C57BL/6L animals carrying a mutant G93A SOD1 (004435, Jackson Laboratory, USA). Colonies were maintained and housed in individually ventilated cages of a constant temperature (25-27°C), and constant humidity (40-50%) for the specific pathogen-free mouse in the Care of Central Animal Services, University of Manitoba. The B6:SOD1-G93A hemizygous transgenic male mice are a breed with female C57BL/6 mice to produce the offspring homologous mice. At least four generations of these mice were created by breeding C57BL/6 female mice with these animals. Ear biopsies were used to collect DNA for PCR genotyping of transgenic progeny according to a Jackson Laboratory technique. The positive rate was about 40% (hereinafter referred to as SOD1^{G93A} mouse). All experiments animals for this study followed protocols were approved by Animal Care Committee at University of Manitoba. Male and female mice were weighed twice weekly and tested for their reflexes and hindlimb elongation to track the progression of the disease. The male-female ratio in the actual experiments

was 2:1 as some of the males were used for breeding. Animals were randomly assigned to young (50-day-old hSOD1^{G93A}, 135-day-old hSOD1^{WT} mice) and middle-aged groups (135-day-old hSOD1^{G93A}, 17-month-old hSOD1^{WT} mice) (n=10 per group).

3.2.2 DNA genotyping and PCR

A total of 300 μ L of TNES buffer (1 M Tris, pH 8.5, 0.5 M EDTA, 10% SDS, 5 M NaCl, distilled water) containing 20 g/L of Proteinase K (25530049, Thermo Fisher Scientific, Waltham, USA) was used to lyse ear samples overnight at 55 °C. The mixture was then added to an equal amount of phenol and chloroform (1:1) and stirred well. After centrifugation for 15 min at 14,500 rpm, the samples were cleared of debris, and the DNA-rich supernatant was recovered. DNA was pelleted by centrifuging it for 10 min at 14,500 rpm after being precipitated in 95% ethanol at -20°C. After centrifuging, the pellet was rinsed with 70% ethanol and centrifuged again to remove the solvent. Any remaining ethanol was evaporated in the ventilated hood over an hour using tubes. Following this, 30 μ L of distilled water was added to the DNA, and the mixture was stored in a refrigerator at 4°C. The genotype determination was applied via the Polymerase Chain Reaction (PCR) with a Phusion High-Fidelity PCR kit (E05538, New England Biolabs, Ipswich, USA). Appropriate PCR reactions (23 μ L/well) were prepared on ice. 2 μ L of sample DNA was added into each well. PCR reactions were performed with an initial denaturation at 98 °C for three min, followed by 30 cycles of 30 s at 98 °C, 30 seconds at 54 °C, and 30 s at 72 °C. A final extension step at 72°C for 7 min concluded the amplification. The resulting PCR products, stained with gel red, were separated on a 1% agarose gel. The gel was visualized using GeneSys imager software on a G: BOX imager (Syngene, Bangalore, India).

3.2.3 Sacrifice animals and tissue preparation

According to the guidance of Care and Use of Experimental Animals of the Canadian Council on Animal Care, mice were sacrificed humanely as previously described⁴⁰³. Mice were transcardially perfused by phosphate-buffered saline (PBS) at PH 7.4 after euthanizing by isoflurane. The cerebral hemispheres, spinal cord, and liver samples were taken out. Tissues were stored at -80 °C or homogenized with an electric homogenizer in ice-cold NP40 lysis buffer (J60766.AP, ThermoFisher Scientific, Waltham, USA) with a cocktail of inhibitors (PI78425, Thermo Fisher Scientific, Waltham, USA). The homogenization was further rinsed twice with another 2 x 300 μ L lysis buffer. The protein samples were placed on an orbital shaker in the 4°C fridge to maintain constant agitation for 30 min. The samples were centrifuged for 20 min at 12,000 rpm at 4°C in a microcentrifuge. The supernatant was gently aspirated from the EP tube on ice and transferred to a new 1.5mL EP tube stored at -80 °C or performed for further experiments.

3.2.4 Cell culture

The motor neuron cell line (NSC34) was purchased from CELLutions Biosystems Inc. (Catalogue: CLU140, Burlington, Canada). As previously described⁴⁵³. Cells were seeded at 5×10^4 cells/ml in 500 μ L of DMEM culture medium (11960069, ThermoFisher Scientific, Waltham, USA) and cultured on glass coverslips in 24-well plates at 37 °C in a humidified incubator with 5% CO₂. When grown at 70-90% confluence, cells were transfected with hSOD1^{G93A} or hSOD1^{WT} plasmid (500ng total DNA/ 2×10^5 cells) with lipofectamine 2000 (11668, Invitrogen, Waltham, USA) for 48 h. Next, cells were digested with trypsin and seeded into a 6-well plate with fresh media containing G418 Sulfate Solution (MIR 5920, Mirus Bio LLC, Madison, USA) at 0.4 mg/mL to selectively kill the non-transfected cells for 7 days. Then, cells were seeded into a 100 mm dish at 1×10^5 cells/ml in a 5ml routine culture medium. The next day, cells were picked and digested

with trypsin from a single clone into a 6-well plate. When cells were more than 70% confluent, they were transferred into a T25 flask for further experiments. The positive transfected cells were identified by anti-hSOD1 antibody (ab52950, Abcam, Waltham, USA) by Western blot.

3.2.5 Immunofluorescence staining

As previously described ⁴¹¹, coverslips were seeded with cultured cells at 5×10^4 cells/ml in 500 μ L of DMEM culture medium. The slides were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) at room temperature for 15 min. After permeabilization with 0.25% Triton X-100 in PBS for 10 min, the cells were blocked with 1% bovine serum albumin in PBST for 30 min. The primary antibody anti-PDH (1:1000, ab110333, Abcam, Waltham, USA) and anti-TOM20 (1:1000, ab186735, Abcam, Waltham, USA) were diluted with 1% bovine serum albumin in PBST and added onto the samples overnight at 4°C. After washing with PBS, the secondary antibody in 1% BSA/PBST were incubated at room temperature for 1 h in the dark. After washing, the nuclei were stained with Hoechst 33258 (94403, Sigma, Virginia Beach, USA) for 5 min, followed by three times of washing. Finally, the coverslips were mounted with 6 μ L of fluorescent mounting medium (S3023, Dako, Carpinteria, USA). Slides were observed under a Z1 microscope or stored at -20°C.

3.2.6 Cell transfection and MitoTracker dye staining in live cells

The plasmid (YFP-STX17) was kindly provided by Dr. Edward A. Fon's Lab (McGill University, Montreal). NSC34 cells (hSOD1^{G93A} or hSOD1^{WT}) were plated at 5×10^4 cells/mL in a live-cell chamber (155383, ThermoFisher Scientific, Waltham, USA) and incubated overnight as previously described ⁴¹¹. Cells were then transfected with the YFP-STX17 with lipofectamine 2000 (11668027, ThermoFisher Scientific, Waltham, USA) for 24 hours. Remove the medium from the chamber and add the prewarmed (37°C) growth medium containing the MitoTracker Red (M22426, Invitrogen, Waltham, USA), as previously described ⁴⁵⁴. Incubate the cells for 15 minutes under

growth conditions. Then replace the loading solution with fresh prewarmed medium and observe the cells using a confocal microscope at 37 °C in a humidified incubator with 5% CO₂.

3.2.7 MitoSOX staining

Cells were seeded in a 24-well plate at 5×10^4 cells/ml in 500 μ L of DMEM culture medium overnight. After washing with PBS, cultured cells were incubated with 5 μ M MitoSOXTM reagent working solution (M36008, Invitrogen, Waltham, USA) and Hoechst 33258 (94403, Sigma-Aldrich, Inc., Burlington, USA) for 10 min as previously described ⁴¹¹. As soon as the staining was completed, it was replaced by fresh prewarmed media or buffer. An imaging microscope with a Zeiss D1 fluorescence system was used to observe live cells, and an imaging microscope with a Zeiss Z1 fluorescence system was used to observe fixed cells.

3.2.8 JC-1 mitochondrial membrane potential assay

Cells were seeded in a 24-well plate at 5×10^4 cells/ml in 500 μ L of DMEM culture medium overnight. After washing with PBS, cultured cells were incubated with 1.0 μ g/mL JC-1 solution (T3168, Invitrogen, Waltham, USA) at 37°C for 20 min as previously described ⁴¹¹. As soon as the staining solution was completed, it was replaced by fresh prewarmed PBS. An imaging microscope with a Zeiss D1 fluorescence system was used to observe live cells.

3.2.9 Reconstruction of MDVs

Mitochondria were isolated from the mouse liver shown in the supplementary figure as previously described ²²⁷. The chest was opened after the animal was deeply anesthetized with isoflurane. Cardiac perfusion was performed with 4 °C saline, and the liver was excised and separated into 380mg per group. After the liver was cut into small pieces, it was homogenized in a cold isolation buffer (20 mM HEPES, pH 7.4, 220 mM mannitol, 68 mM sucrose, 76 mM KCl, 4 mM KOAc, and 2 mM MgCl₂, supplemented with protease inhibitors benzamidine, PMSF, aprotinin, and

leupeptin). Post-nuclear supernatants were collected by centrifugation at 600 g for 10 min with a homogenate (J-25I, Beckman Coulter, Inc., Brea, USA). Mitochondrial pellets were further isolated from the supernatant by centrifugation at 7,000 g for 10 min. And they were resuspended and centrifuged with 2 mL of isolation buffer for washing and then stored on ice in isolation buffer with protease inhibitor. A 90-Ti rotor was used to ultracentrifuge the supernatant for 90 min at 200,000 g in an ultracentrifuge. Afterwards, the supernatant was mixed with mitochondria fraction in the presence of 50 μ M antimycin A and an ATP regenerating mixture (1 mM ATP, 5 mM succinate, 80 μ M ADP, and 2 mM K₂HPO₄, pH 7.4)²²⁷. A 2 h incubation was carried out at 37 °C (vertexing every 30 minutes), followed by centrifugation at 10,000 g for 10 min. Transfer the supernatant into a new vial and then recentrifuged again at 10,000 g for another 10 min. The pellet containing mitochondria was resuspended by lysis buffer for further BCA test and Western blot analysis. For protein analysis, the supernatant was ultracentrifuged for 90 min at 200,000 g, and the MDV pellet was resuspended by lysis buffer for Western blot. The supernatant was fixed with 3% glutaraldehyde for electron microscopy staining and then ultracentrifuged for 90 min at 200,000 g. The MDV pellet was then fixed following the protocol of transmission electron microscopy afterwards.

3.2.10 Transmission electron microscopy for isolated MDVs and Mitochondria

The isolated mitochondria were fixed with 3% glutaraldehyde in PBS at room temperature for 3-5 h, as previously described⁵¹. The samples were rinsed by changing at least 3 times the 6% sucrose solution prepared in Sorensen's buffer. Then remove the sucrose and post-fix the mitochondria and MDV pellet in 1% Osmium tetroxide O₃O₄ (19152, Electron Microscopy Sciences, Hatfield, USA) in 0.1M Sorensen's buffer for 2 h. Remove and discard O₃O₄ in a waste container. Rinse three times with 0.1M, 0.05 M Sorensen's buffer, and ddH₂O for 10 min each

time. Dehydrate with 30%, 50%, 70%, 95%, and 100% Ethanol (15058, Electron Microscopy Sciences, Hatfield, USA) and 100% Methanol separately for 120 min. Then the sample was embedded with 100% propylene oxide (20401, Electron Microscopy Sciences, Hatfield, USA) for 20 min, with 1:2 Epon and 2:1 Epon for 1 h separately, and with 100% Epon overnight. The 100% Epon was prepared by mixing the reagents bought from Electron Microscopy Sciences, including EMBed-812 (14900, Electron Microscopy Sciences, Hatfield, USA), DDSA (13710, Electron Microscopy Sciences, Hatfield, USA), NMA (19000, Electron Microscopy Sciences, Hatfield, USA), and DMP-30 (13600, Electron Microscopy Sciences, Hatfield, USA). The samples were placed in desired models filled with 100% Epon in an oven heated at 60 °C for 24-48 h. The samples were trimmed and cut into thin sections (90 nm) by a microtome. Thin sections were then collected onto copper grids and stained with UranylLess (22409, Electron Microscopy Sciences, Hatfield, USA) for 3 min and lead citrate (22410, Electron Microscopy Sciences, Hatfield, USA) for 1 min, followed by 3 times washing in water. Finally, photographs were taken on a Philips CM10 electron microscope at 60 kV.

3.2.11 Transmission electron microscopy for mouse spinal cord

After the mouse was deeply anaesthetized with isoflurane, the chest was opened. Cardiac perfusion was performed with 4 °C saline, and a portion of the spinal cord (L4-L5 segment) was dissected for transmission electron microscopy analysis as previously described⁵¹. The removed spinal cord was fixed in 4% paraformaldehyde at 4 °C overnight, followed by treatment with 10%, 20%, and 30% sucrose solution at 4 °C for 24 hours. Then, the tissue was fixed with 3% glutaraldehyde in PBS at room temperature for 3-5 hours and rinsed by changing at least 3 times the 6% sucrose solution prepared in Sorensen's buffer. Then the tissues were post-fixed in 1% osmium tetroxide OsO_4 (19152, Electron Microscopy Sciences, Hatfield, USA) in 0.1M Sorensen's buffer for 2 hours.

After the O_3O_4 was discarded into a waste container, the tissues were rinsed three times with 0.1M, 0.05 M Sorensen's buffer, and ddH₂O for 10 min each time. The tissue blocks were dehydrated with 30%, 50%, 70%, 95%, and 100% Ethanol (15058, Electron Microscopy Sciences, Hatfield, USA) and 100% Methanol separately for a total of 120 min. Then the tissue blocks were embedded with 100% propylene oxide (20401, Electron Microscopy Sciences, Hatfield, USA) for 20 min, with 1:2 Epon and 2:1 Epon for 1 hour separately, and with 100% Epon overnight. The 100% Epon was prepared by mixing the reagents bought from Electron Microscopy Sciences, including EMBed-812 (14900, Electron Microscopy Sciences, Hatfield, USA), DDSA (13710, Electron Microscopy Sciences, Hatfield, USA), NMA (19000, Electron Microscopy Sciences, Hatfield, USA), and DMP-30 (13600, Electron Microscopy Sciences, Hatfield, USA). The samples were placed in desired models filled with 100% Epon in an oven heated at 60 °C for 24-48 hours. The samples were trimmed into thin sections with 90 nm on a microtome. The thin sections were then collected onto copper grids and stained with UranylLess (22409, Electron Microscopy Sciences, Hatfield, USA) for 3 min and lead citrate (22410, Electron Microscopy Sciences, Hatfield, USA) for 1 min, followed by 3 times washing in water. Finally, photographs were taken on a Philips 201 electron microscope.

3.2.12 Western blot

Tissues were homogenized, and cells were broken in the RIPA lysis buffer (BP571-5, ThermoFisher, Waltham, USA) as previously described ⁴⁰⁵. The protein concentrations were measured using a BCA protein assay kit (23225, ThermoFisher, Waltham, USA). Afterwards, the homogenates were electrophoretically separated using SDS-PAGE. Proteins were separated by electrophoresis and transferred to PVDF membranes, where they were blocked in the blocking buffer (5% milk in PBST) for an hour. A primary antibody was then incubated overnight at 4°C

on the membrane, followed by a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. An ECL kit was used to visualize the antigen-antibody complexes (1705061, Bio-Rad, Hercules, USA). Stain-free total protein was applied as loading control ⁴⁰⁸⁻⁴¹⁰.

Table 3 Primary Antibodies

Antibody	Cat#	Host	Application	Dilution	Company
PDH	ab110333	Mouse	IF, WB	1:1000	Abcam, Waltham, USA
TOM20	ab186735	Rabbit	IF, WB	1:1000	Abcam, Waltham, USA
Syntaxin 17	ab229646	Rabbit	WB	1:500	Abcam, Waltham, USA
Lamp2	sc18822	Mouse	IF	1:500	Santa Cruz, Dallas, USA
PDI	3501	Rabbit	WB	1:1000	Cell Signaling, Danvers, USA
Calnexin	2679	Rabbit	WB	1:1000	Cell Signaling, Danvers, USA
RCAS1	12290	Rabbit	WB	1:1000	Cell Signaling, Danvers, USA
Syntaxin 6	2869	Rabbit	WB	1:1000	Cell Signaling, Danvers, USA
COX4	ab16056	Rabbit	WB	1:1000	Abcam, Waltham, USA
β -actin	sc47778	Mouse	WB	1:5000	Santa Cruz, Dallas, USA

3.2.13 Statistical analysis

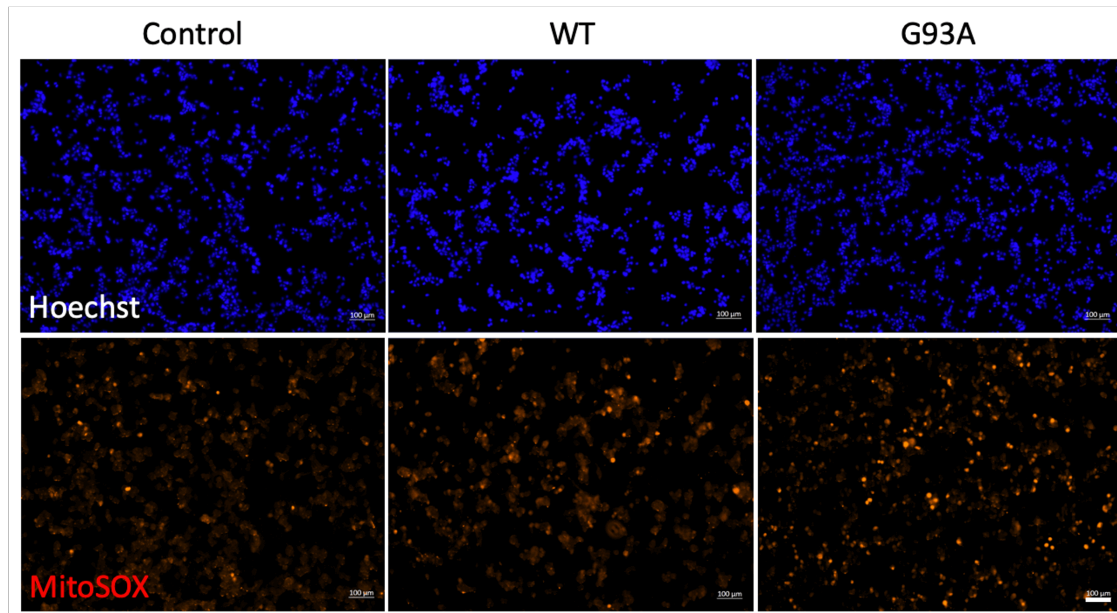
Statistical analysis of the data was performed using Prism9. Data were expressed as mean $\pm$ standard error of the mean (SEM). An unpaired t-test was applied to compare differences between the control and experimental groups. Multiple comparisons were performed by one-way ANOVA followed by Bonferroni's test. Differences were considered significant when * $p < 0.05$, ** $p < 0.01$.

3.3 Results

3.3.1 Mitochondrial dysfunction in aging

Mitochondria serve as the primary site to produce ROS. Although the majority of SOD1 is expressed in the cytosol, a fraction of SOD1 monomers can translocate into the mitochondrial intermembrane space (IMS) ³¹¹. SOD1, especially mutant SOD1, is vulnerable to forming protein aggregation which may trigger mitochondrial dysfunction. We have previously proven that NSC34^{G93A} cells exhibit senescent phenotypes after 3 weeks of transfection. To assess mitochondrial function in senescent cells, we initially measured mitochondrial superoxide using MitoSOX. As depicted in Figure 3.1, senescent NSC34^{G93A} cells exhibited significantly higher ROS production compared to NSC34^{WT} and non-transfected control cells.

(a)



(b)

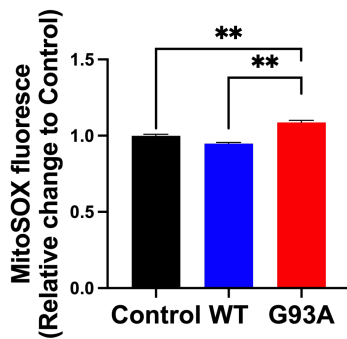
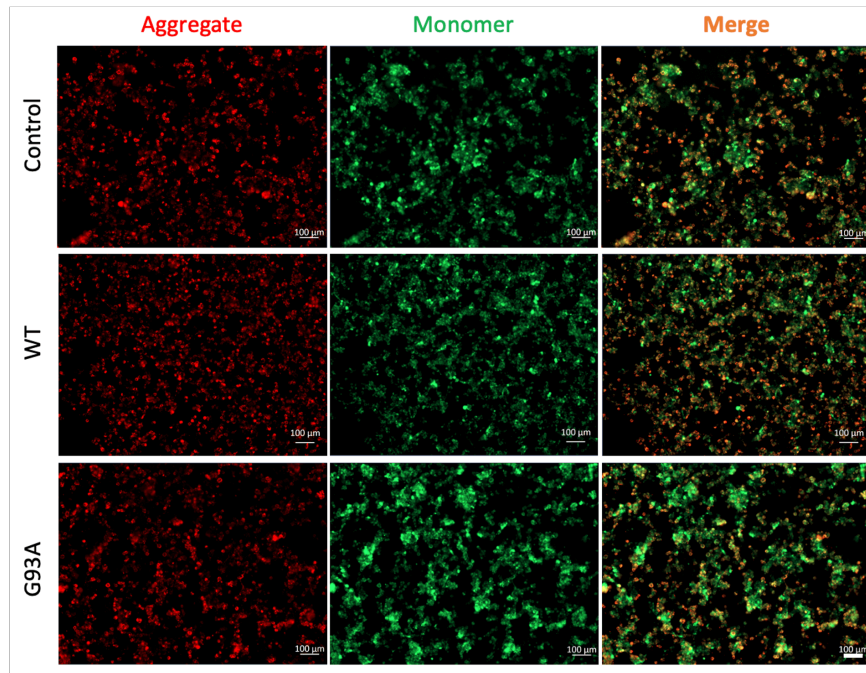


Fig.3.1 Mitochondrial ROS production increases in NSC34^{G93A} cells

(a) Mitochondrial ROS was detected with MitoSOX (red), and the nucleus was stained with Hoechst (blue) in non-transfected(control), WT, and G93A transfected NSC34 cells. Scale bar, 100 μm. (b) Quantification of relative fluorescence intensity of MitoSOX (n= 260 cells, mean ± SEM, ** $p < 0.01$)

Subsequently, we evaluated mitochondrial function in senescent cells by employing the JC-1 dye to measure mitochondrial membrane potential ($\Delta\Psi_m$). JC-1 monomer fluoresces green, and its aggregated form fluoresces red. Therefore, the JC-1 assay is based on the ability of the JC-1 dye to undergo a reversible color shift in response to changes in mitochondrial membrane potential. the ratiometric measurement of red to green fluorescence of JC-1 to assess changes in mitochondrial membrane potential. In healthy cells with an intact $\Delta\Psi_m$, JC-1 accumulates in the mitochondria, forming aggregates with red fluorescence. In cells with a decreased $\Delta\Psi_m$ (indicative of mitochondrial dysfunction or apoptosis), JC-1 remains in its monomeric form with green fluorescence. In this experiment, the quantification of mitochondrial membrane potential was performed in hSOD1^{G93A}, hSOD1^{WT} and non-transfected NSC34 cells. Our findings revealed that the mitochondria in NSC34^{G93A} cells exhibited depolarization, indicated by a 15.45% reduction in membrane potential compared to non-transfected NSC34 cells. However, NSC34^{WT} cells maintained a stable red/green fluorescence ratio, indicating preserved mitochondrial membrane potential (Figure 3.2).

(a)



(b)

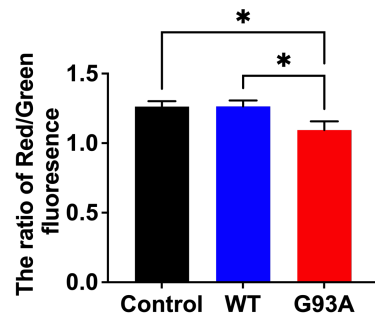


Fig.3.2 Mitochondrial depolarization is found in NSC34^{G93A} cells

(a) Detection of JC-1 aggregates (red) and monomers (green) in non-transfected (control), WT, and G93A transfected NSC34 cells. Scale bar, 50 μm. (b) Quantitative analysis of the ratio of aggregates and monomers of JC-1 (n=15, mean ± SEM, * $p < 0.05$)

Besides, mitochondria with obvious elongated and vacuolated morphology were also found in NSC34^{G93A} cells using EM (Fig. 3.3). Furthermore, we assessed mitochondrial biogenesis through Western blot analysis, which revealed the protein level of PGC-1 α decreased by a 72.85% in NSC34^{G93A} cells and 36.73% in NSC34^{WT} cells compared with non-transfected NSC34 cells. Additionally, there was a 33.18% increase in the protein level of MFN2 in NSC34^{G93A} cells compared with non-transfected NSC34 cells. These findings suggest impaired mitochondrial biogenesis accompanied by over-fused mitochondria in NSC34^{G93A} cells leading to accelerated accumulation of oxidized hSOD1(Fig. 3.4). Collectively, these findings provide evidence of impaired mitochondrial function in senescent NSC34 cells.

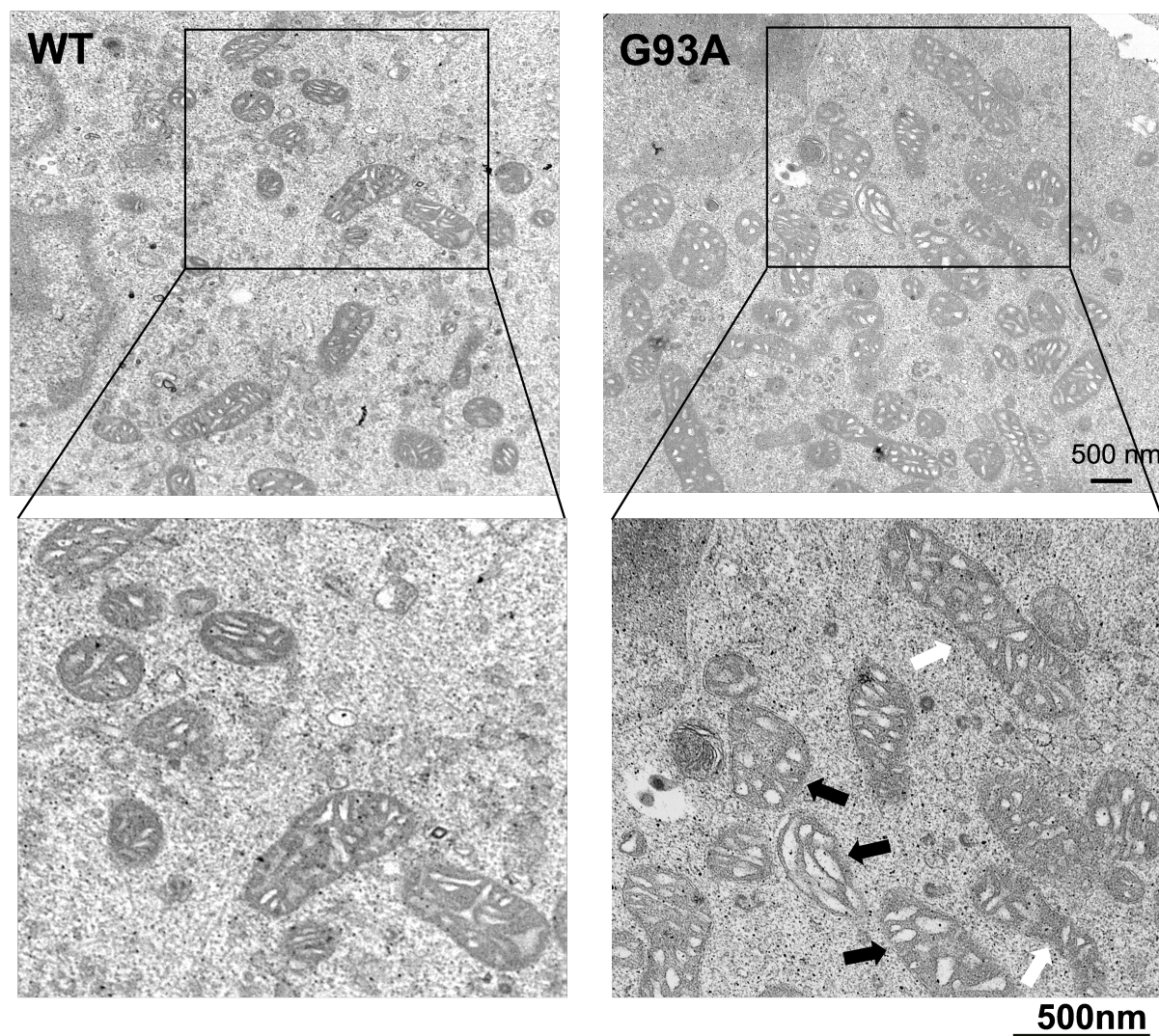


Fig.3.3 Mitochondria with obvious morphologic changes in NSC34^{G93A} cells

Ultrastructure of mitochondria in NSC34^{G93A} cells was observed using TEM. The elongated (white arrow) and vacuolated (black arrow) mitochondria in NSC34^{G93A} cells were compared to NSC34^{WT} cells. Scale bar, 500 nm.

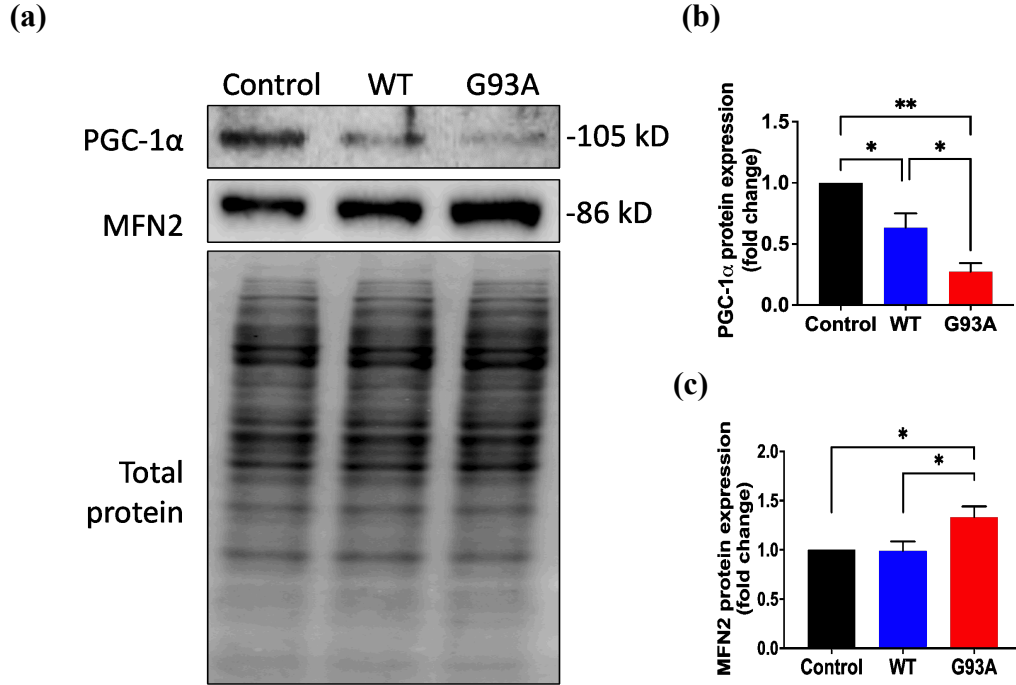
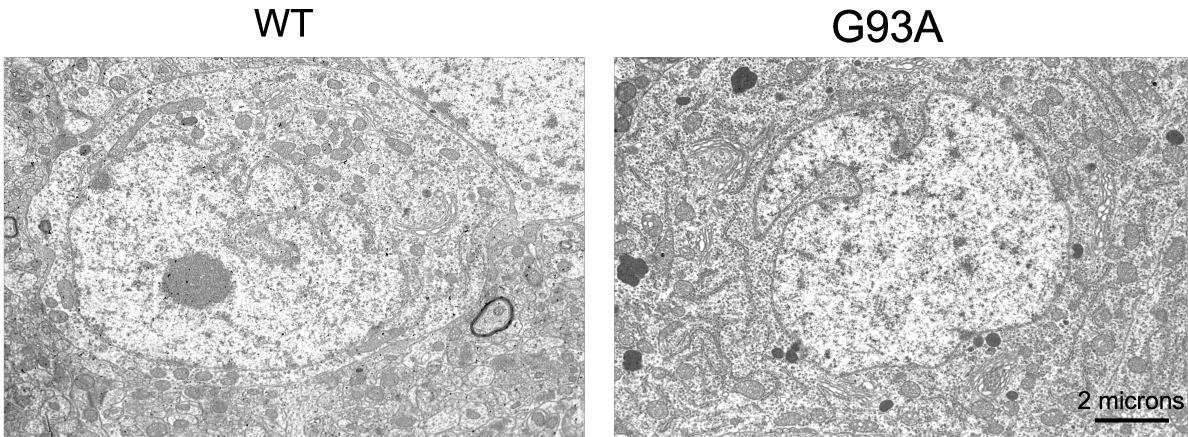


Fig.3.4 Mitochondrial biogenesis and dynamic changes in NSC34 cells

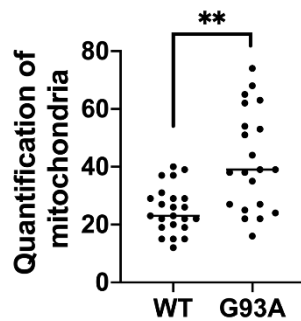
(a) Representative Western blot images of mitochondrial biogenesis proteins (PGC-1 α and MFN2) in NSC34 cells. **(F-G)** Quantitative analysis of PGC-1 α and MFN2 (n=6, mean $\pm$ SEM, * p < 0.05, ** p < 0.01).

To gain further insights into the mitochondrial condition *in vivo*, we employed EM to examine the morphology of mitochondria in spinal cord motor neurons from 135-day hSOD1 transgenic mice (Fig. 3.5a). Our observations revealed that the number of mitochondria in each hSOD1^{G93A} motor neuron was 1.6 times higher compared to hSOD1^{WT} motor neurons (Fig. 3.5b and c). Moreover, we noted that the maximum diameter of mitochondria in hSOD1^{G93A} motor neurons ($0.45 \pm 0.02 \mu\text{m}$) was reduced compared to hSOD1^{WT} motor neurons ($0.5605 \pm 0.02 \mu\text{m}$). These findings suggest a propensity for mitochondrial fission rather than fusion, potentially enabling the elimination of damaged portions of mitochondria in 135-days hSOD1^{G93A} motor neurons.

(a)



(b)



(c)

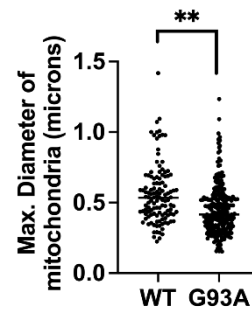


Fig.3.5 The mitochondrial morphology in spinal cord motor neurons from transgenic mice

(a) Representative TEM images illustrate motor neurons of the anterior spinal cord in both hSOD1^{WT} and hSOD1^{G93A} mice at 135 days. Scale bar, 2 μ m. (b) Quantification of the number of mitochondria in each motor neuron (n=20, mean $\pm$ SEM, ** $p < 0.01$). (c) Quantification of the size of mitochondria with the index of maximum diameter (n=150, mean $\pm$ SEM, ** $p < 0.01$).

In addition, we conducted a molecular experiment to investigate mitochondrial biogenesis in brain tissue (Fig. 3.6a). Our results demonstrated that the protein levels of both PGC-1 α and MFN2 were

decreased in the 135-days G93A transgenic mice compared to the same age of WT mice (Fig. 3.6b and c). These findings further validate the morphological observations and support the notion of reduced mitochondrial synthesis coupled with enhanced mitochondrial segregation as part of the mitochondrial quality control mechanisms.

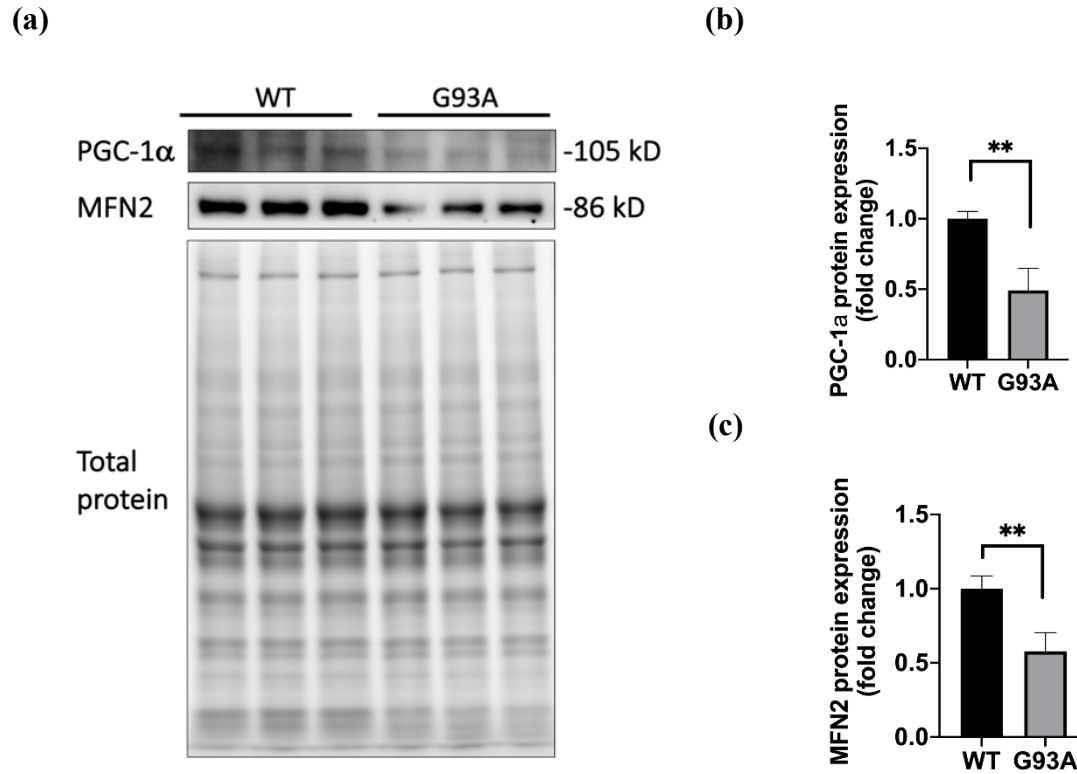


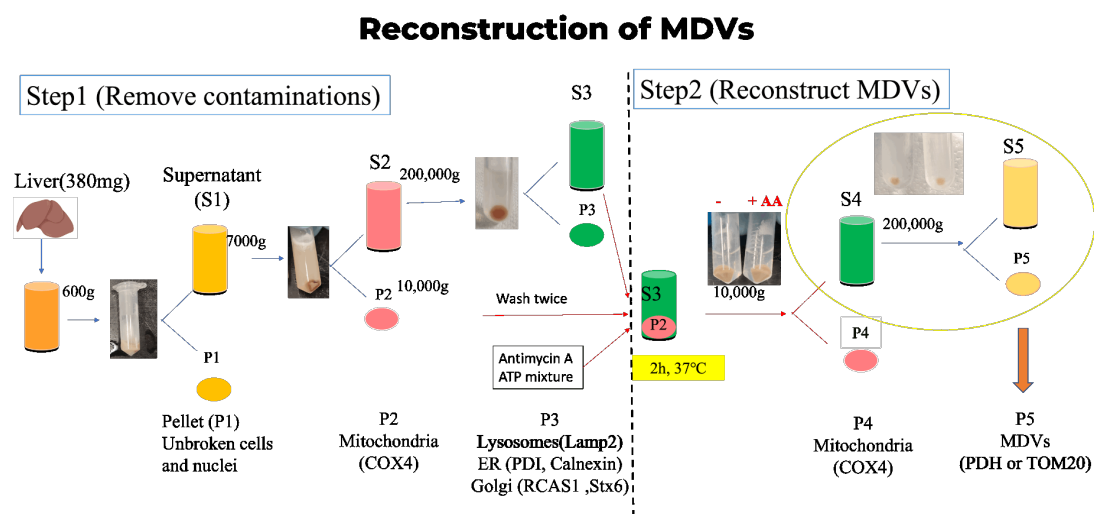
Fig.3.6 Mitochondrial biogenesis declines in the brain tissue of hSOD1^{G93A} transgenic mice

(a) Western blot analysis was conducted to examine the protein levels of key mitochondrial biogenesis markers, PGC-1α and MFN2. **(b, c)** The protein expressions of PGC-1α and MFN2 were quantified (n=3) to provide a quantitative assessment of their abundance. Statistical analysis was performed using a student's t-test. The level of significance was set at **p<0.01.

3.3.2 Oxidized hSOD1-enriched MDVs increase in early aging

Previous studies have demonstrated mitochondrial dysfunction *in vitro* and *in vivo*, which aligns with previous research ⁴⁵⁵. To explore mitochondrial quality during the early stages of aging, we directed our attention towards MDVs, which play a crucial role in the initial phases of MQC. The experimental workflow is illustrated in Figure 3.7 and different age groups are illustrated in Fig.2.12a.

(a)



(b)

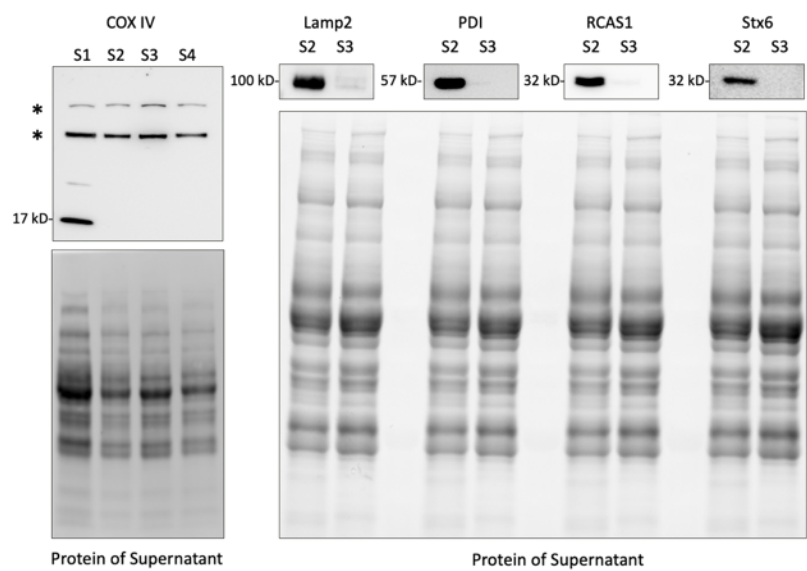


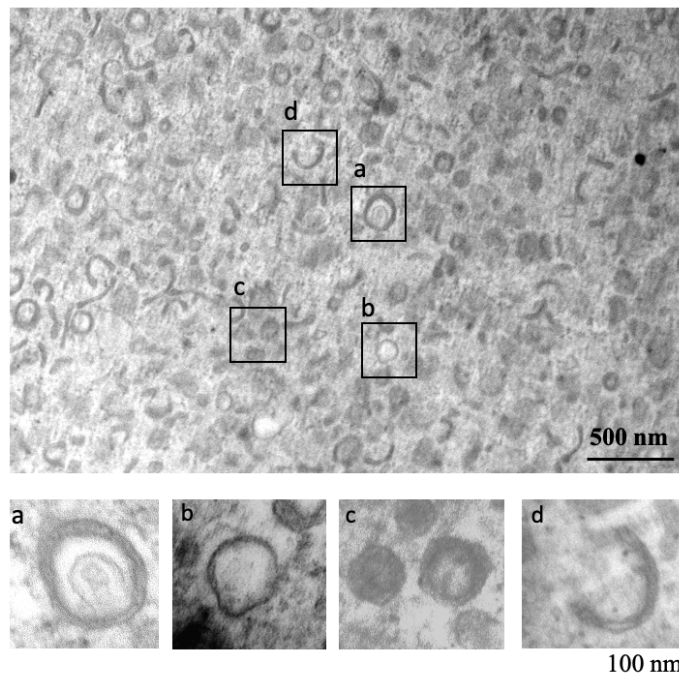
Fig.3.7 The flow chart depicts two major steps for the reconstruction of MDVs

(a) Presented is a flowchart outlining the two primary phases involved in the reconstruction of MDVs. Initially, MDVs were extracted from 380 milligrams of liver tissue. In the first phase, mitochondria were isolated from the homogenized liver, and potential contamination from other organelles was eliminated through ultracentrifugation. The subsequent phase involved MDV reconstruction by co-incubating mitochondria and cytosol in the challenge of antimycin A and an ATP regeneration mixture. To obtain purified MDVs, the supernatant underwent ultracentrifugation for 90 minutes at 200,000 g. The MDV pellet was then either resuspended using RIPA lysis buffer for Western blot analysis or fixed for electron microscopy. **(b)** Each stage of the isolation process was rigorously validated for purity, with a focus on eliminating contamination. This assessment was conducted by examining specific biomarkers for mitochondria (COX4), lysosomes (Lamp2), endoplasmic reticulum (PDI, Calnexin), and Golgi (RCAS1, Stx6) using Western blot analysis. The asterisk (*) indicates the presence of a non-specific band.

Based on our earlier observations indicating the presence of oxidized hSOD1 and mitochondrial proteins (PDH and TOM20) in EVs derived from neural stem cells. To investigate the role of MDVs, we isolated MDVs from liver mitochondria. MDVs were further confirmed with EM, revealing structures with single or double membranes and diameters ranging from 70 to 150 nm (Fig. 3.8a). Remarkably, we found that hSOD1 was present in all MDV proteins, and its levels were elevated in both middle-aged MDVs expressing hSOD1^{WT} (MDV^{WT}) and hSOD1^{G93A} (MDV^{G93A}). By employing the specific A5C3 antibody, we successfully detected misfolded SOD1 in the aged MDV^{WT} and MDV^{G93A}. Specifically, MDV^{WT} exhibited partial aggregation at higher and lower bands, while hSOD1 in MDV^{G93A} appeared as fully aggregated and exclusively at the higher band (Fig. 3.8b). These findings highlight the propensity of hSOD1 within MDVs to form high molecular weight of proteins in aged hSOD1^{WT} and hSOD1^{G93A} mice.

We further quantified MDVs using Western blot analysis based on the protein levels of PDH and TOM20 (Fig. 3.9a). The results revealed increased production of PDH⁺ and TOM20⁺ MDVs in middle-aged mitochondria compared to young mitochondria (Fig. 3.9b-e), indicating an augmentation of oxidized hSOD1-containing MDVs during early aging.

(a)



(b)

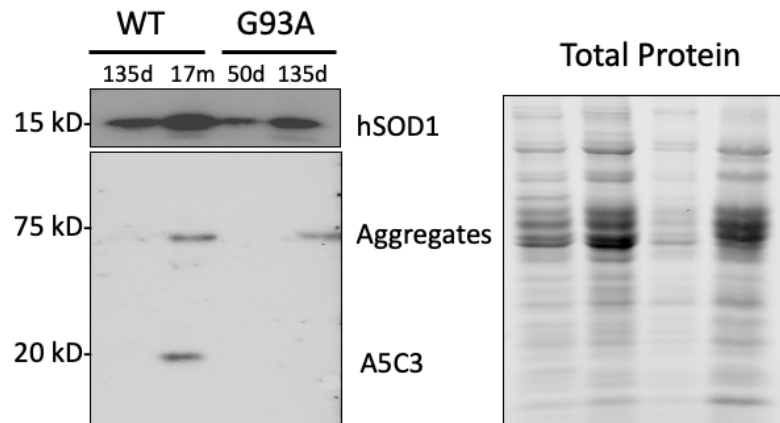
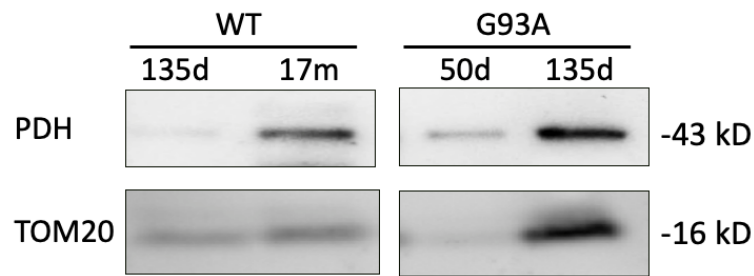


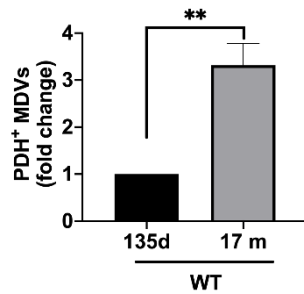
Fig.3.8 Isolated MDVs contain a high amount of misfolded and aggregated hSOD1

(a) The ultrastructure of isolated MDVs from liver mitochondria by using TEM. Upper: lower magnification of MDV pellet. Scale bar, 500 nm. Lower: higher magnification of MDV with double membranes, single membrane, dense core, or crescent shape. Scale bar, 100 nm. (b) Representative Western blot images of hSOD1 and misfolded SOD1 (A5C3) in young and middle-aged MDVs isolated from G93A and WT liver mitochondria.

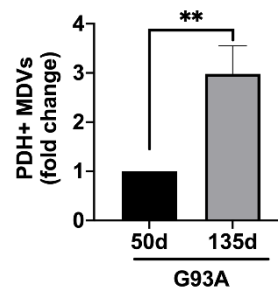
(a)



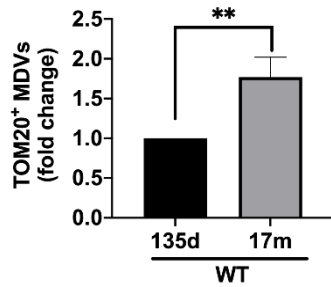
(b)



(c)



(d)



(e)

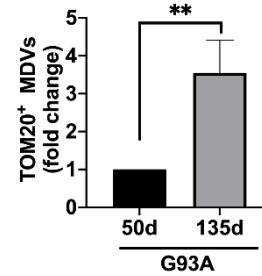


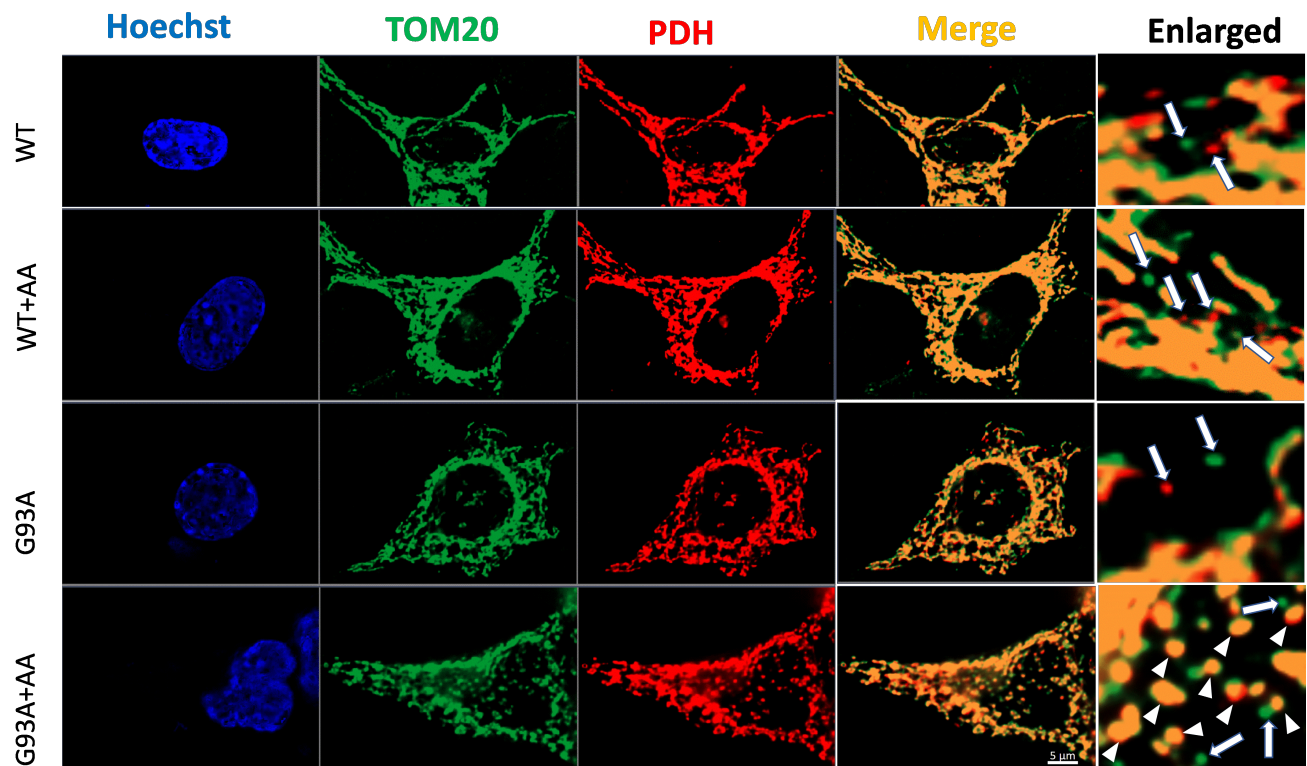
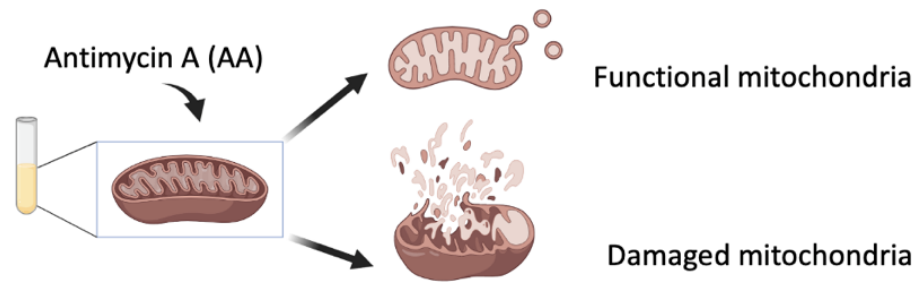
Fig.3.9 Aged mitochondria generate more MDVs than young mitochondria

(a) Representative Western blot images of mitochondrial biogenesis proteins (PGC-1 α and MFN2) in brain tissue from 135-day hSOD1 transgenic mice. (b-e) Quantitative analysis of PGC-1 α and MFN2 (n=6, mean $\pm$ SEM, ** p < 0.01).

3.3.3 Senescent cells exhibit a reduced mitochondrial responsiveness in early aging

To evaluate mitochondrial responsiveness to oxidative stress, we subjected hSOD1^{G93A} and hSOD1^{WT} cells to mild oxidative stress induced by 10 μ M Antimycin A for 2h. NSC34^{WT} cells retained their network structures and generated more MDVs when challenged with AA compared to the unchallenged NSC34^{WT} cells. Conversely, senescent NSC34^{G93A} cells exhibited mitochondrial fragmentation after AA challenge (Fig. 3.10a). Furthermore, we transfected NSC34 cells with YFP-STX17, a transporter of MDVs, and stained mitochondria with MitoTracker Red for live cell imaging (Fig. 3.10b). Due to the lateral resolution of the microscope, 70-150 nm MDVs are found with a diameter <300 nm under confocal microscope. In the 10 min time-lapse images, we observed several YFP-STX17 positive MDVs colocalizing with MitoTracker Red in NSC34^{WT} cells, whereas STX17-aggregates were observed in senescent NSC34^{G93A} cells. This finding suggests that senescent NSC34^{G93A} cells exhibit impaired responsiveness to mild oxidative stress.

(a)



(b)

YFP-STX17 MitoTracker Red

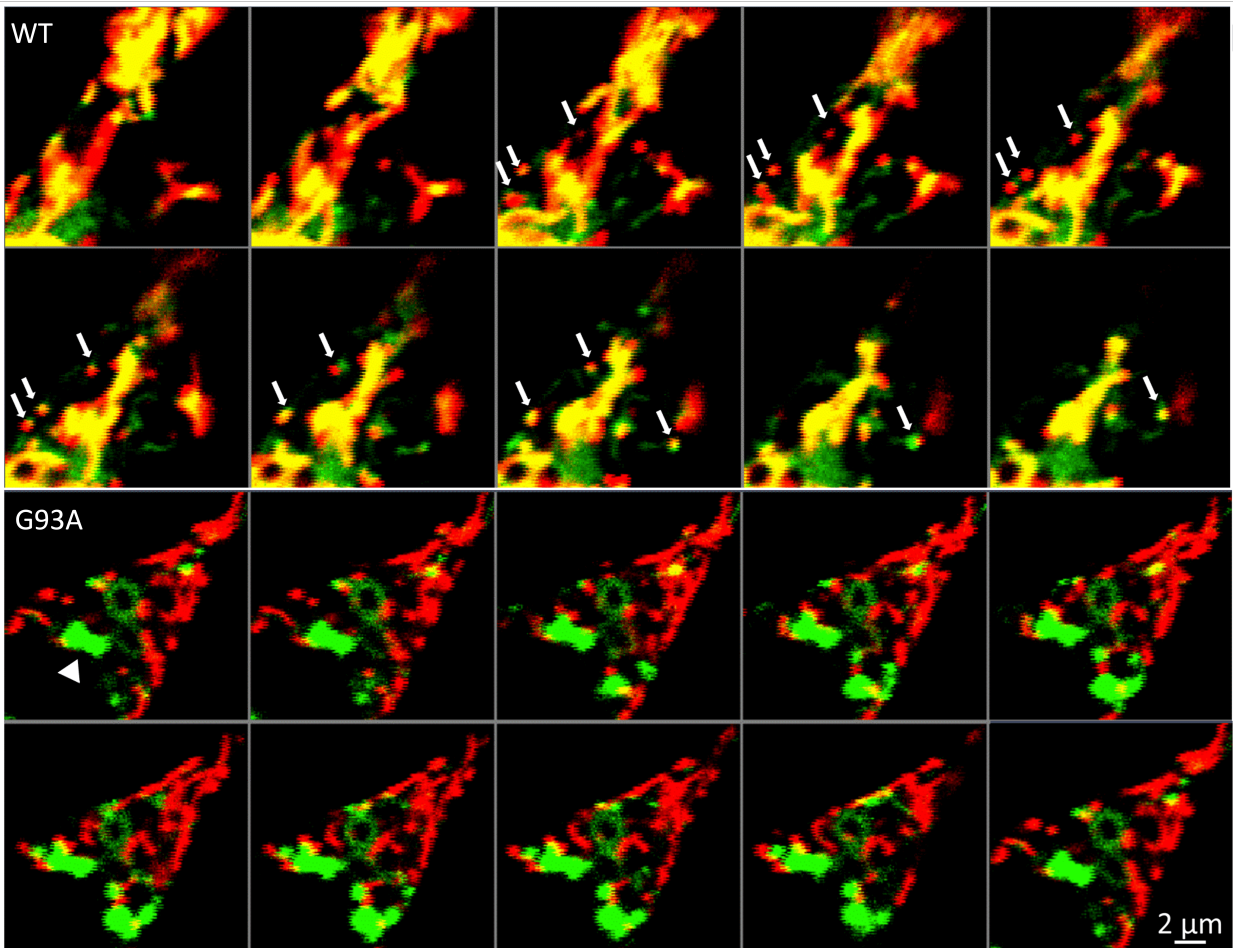


Fig.3.10 Mitochondrial responsiveness decreases in NSC34^{G93A} cells

(a) Immunofluorescence staining of MDVs generated from mitochondria with or without the challenge of Antimycin A in NSC34^{WT} and NSC34^{G93A} cells. Arrows indicate MDVs are positive for either PDH (red) or TOM20 (green). Arrowheads indicate fragmented mitochondria with colocalization of PDH and TOM20. Scale bar, 5 μ m. (b) Representative live cell confocal imaging showing MDVs (~300nm) stained with MitoTracker Red (red) are colocalized with YFP-STX17 (green) in 10-minute time-lapse images. Arrows indicate MDVs in NSC34^{WT} cells, and the

arrowhead indicates aggregation of STX17 (green aggregation, >2 μ m) in NSC34^{G93A} cells. Scale bar, 2 μ m.

To investigate the responsiveness of mitochondria during early aging stages, we collected mitochondria from the livers of young and middle-aged hSOD1 transgenic mice. Subsequently, we reconstructed MDVs from these mitochondria, with or without challenge of AA and examined their morphology using EM (Fig. 3.11a and b). MDVs, with a diameter of 70-150 nm were observed adjacent to mitochondria. We observed an increase in the ratio of mitochondria with MDVs following AA challenge in the young group. However, aberrant MDVs were observed in the AA-challenged middle-aged group. The ratio of mitochondria with MDVs to the total amount of mitochondria per EM image field was significantly increased in AA challenged young group than unchallenged group. However, the differences between young and middle-aged groups were not statistically significant (Fig. 3.11c and d). These findings suggest a decline in mitochondrial responsiveness with age, regardless of whether the mitochondria originated from hSOD1^{G93A} or hSOD1^{WT} mice.

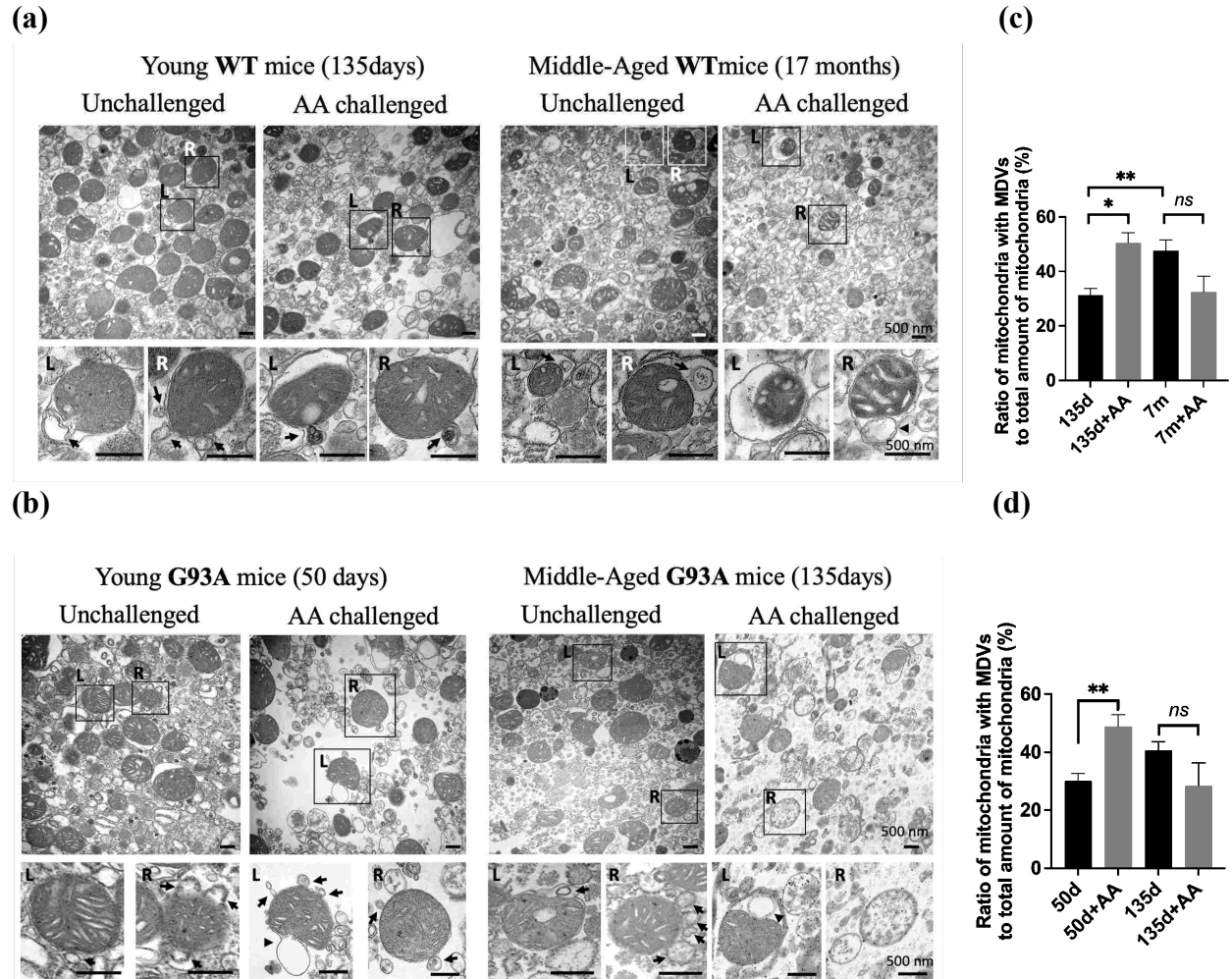
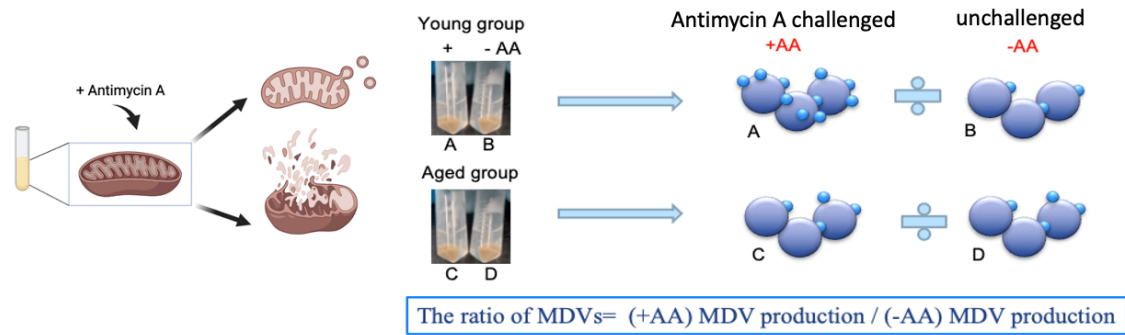


Fig.3.11 Mitochondrial morphology in young and middle-aged mice

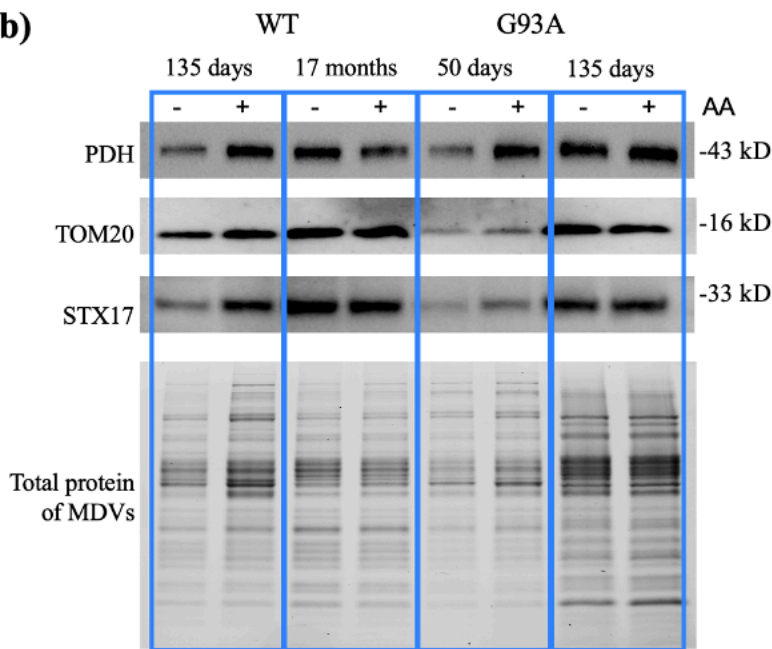
(a-b) Representative TEM images showing isolated liver mitochondria from young and middle-aged **(a)** hSOD1^{WT} and **(b)** hSOD1^{G93A} transgenic mice. Black arrows indicate MDVs (70-150 nm) and arrowheads indicate autophagosomes (>500 nm). Scale bar, 500 nm. **(c-d)** Quantification of the ratio of mitochondria with MDVs to the total amount of liver mitochondria from **(c)** hSOD1^{WT} and **(d)** hSOD1^{G93A} transgenic mice.

Mitochondrial responsiveness was determined by the ratio of MDVs with or without the challenge of AA (Fig. 3.12a). To assess mitochondrial responsiveness at the molecular level, we examined biomarkers of MDVs (PDH, and TOM20) and MDV transporter (STX17) using Western blot (Fig.3.12b). The data revealed a significant decrease in responsiveness of mitochondria in middle age (135 days for the G93A group and 17 months for the WT group) compared to young age (50 days for the G93A group and 135 days for the WT group) (Fig. 3.12c-d). These findings indicate a decline in mitochondrial responsiveness during early aging.

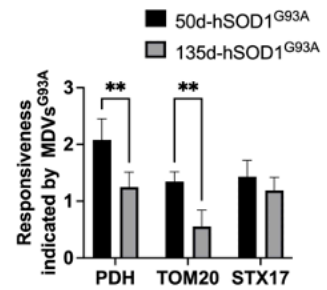
(a)



(b)



(c)



(d)

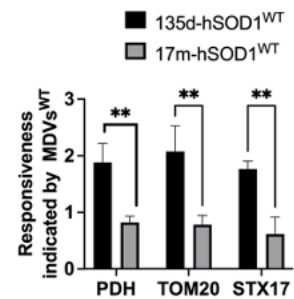


Fig.3.12 Mitochondrial responsiveness declines in middle-aged mitochondria

(a) Representative Western blot images of protein level of MDVs isolated from hSOD1^{WT} and hSOD1^{G93A} mice liver. (b-d) Quantification of the protein levels of MDVs (PDH, TOM20, and STX17) (n=3, mean $\pm$ SEM. ** $p < 0.01$).

3.4 Discussion

Mitochondria have crucial roles in bioenergetics, ROS production, anabolism and catabolism, iron-sulfur cluster and heme formation, calcium and iron homeostasis, apoptosis, and signal transmission, making them pivotal regulators of cellular metabolism and homeostasis ⁴⁵⁶. In response to cellular stress, these organelles are dynamic and reprogram metabolism ⁴⁵⁷. Mitochondrial dysfunction in the aging process is characterized by various factors, including impaired oxidative phosphorylation (OXPHOS) activity, increased oxidative damage, declined mitochondrial quality control, decreased activity of metabolic enzymes, and alterations in mitochondrial morphology, dynamics, and biogenesis ⁴⁵⁸. These interconnected processes contribute to the overall decline in mitochondrial function observed during aging ⁴⁵⁹. Age-related disorders like neurodegenerative and cardiovascular diseases, diabetes, obesity, and cancer have all been linked to mitochondrial dysfunction ¹¹⁴. However, the mechanisms through which mitochondrial function modulates the aging process remain unclear.

In our study, we found high production of ROS and mitochondrial depolarization in senescent NSC34^{G93A} cells. Previous studies have notably emphasized the presence of megamitochondria within neuromuscular junction axons in aged rats ⁴⁵⁵. Additionally, approximately 10% of ALS patients displayed ultrastructural abnormalities in muscle mitochondria within skeletal muscle tissue, including giant mitochondria, protein inclusions, and abnormal cristae ⁴⁶⁰. A significant mitochondrial vacuolation in motor neurons is also indicative of an advanced disease stage ¹⁴⁵. In this study, we conducted further observations of NSC34 cells expressing hSOD1^{G93A} using TEM. In senescent NSC34^{G93A} cells, mitochondria appeared larger, elongated, and vacuolated compared to NSC34^{WT} cells. Additionally, we assessed the protein levels within the mitochondria and observed a downregulated level of PGC1 α but an upregulated level of MFN2. All these findings

collectively suggest that mitochondrial fusion predominates in senescent NSC34^{G93A} cells, which have been proven to contain high levels of oxidized SOD1. These over-fused mitochondria may further lead to the accumulation of aberrant mitochondria and accelerate cellular senescence.

Subsequently, the question arises: What occurs within mitochondria during the early stages of aging? A previous study addressed this by examining corticospinal motor neurons at an early stage (specifically, post-natal day 15). They found motor neurons displayed mitochondrial defects and extensive enlargement, while non-corticospinal motor neurons remained healthy ⁴⁶¹. In contrast, our research in this study focused on corticospinal motor neurons in middle-aged (135-day-old) hSOD1^{G93A} mice without disease symptom development, revealing a distinct pattern. Here, we observed a higher quantity of mitochondria with a smaller diameter in hSOD1^{G93A} mice compared to their hSOD1^{WT} counterparts. These findings suggest a preference for mitochondrial fission over fusion during middle age, potentially implying the regenerative capability of middle-aged mitochondria in eliminating damaged counterparts within hSOD1^{G93A} motor neurons.

In addition, the protein level of PGC-1 α was found to gradually decrease in the process of aging, suggesting a gradually declined mitochondrial biogenesis. Whereas the protein level of MFN2 decreases in early aging but increases in the late stage of aging. These findings imply that mitochondria are actively involved in segregating damaged components during the early stages of aging, but their capacity to perform this function diminishes with advanced age.

MQC system protects mitochondria by maintaining organelle flexibility and facilitating interactions between mitochondria and other cellular components ⁴⁶². Mitochondria communicate with the endosomal and lysosomal compartments to keep cellular homeostasis ^{463,464}. Mitochondria should be adaptable and maintain connections with different cellular compartments to ensure efficient energy provision and the proper integration of intracellular signals ⁴⁶⁵. Recent studies

have found mild oxidative stress stimulates the packing of oxidized mitochondrial proteins into single or double-membrane MDVs, ultimately targeted for destruction by lysosomes²³⁶. As an earlier MQC, MDV formation could deliver oxidized proteins to lysosomes and peroxisomes for degradation²¹⁷. However, the involvement of MDVs in the aging process remains poorly understood, and there is insufficient evidence to elucidate their specific role. As a result, further research is needed to investigate the mechanisms by which MDVs contribute to aging and to gain a deeper understanding of their impact on cellular and mitochondrial function during the aging process. In our *in vitro* study, we discovered that multiple MDVs were generated adjacent to the main mitochondrial structure in NSC34^{WT} cells, while senescent NSC34^{G93A} cells exhibited a significantly lower number of MDVs. Furthermore, when subjected to mild oxidative stress challenged by antimycin A, mitochondria in NSC34^{WT} cells were stimulated to promote MDV formation, whereas mitochondria in NSC34^{G93A} cells experienced severe damage. These results strongly suggest that senescent cells have lost their capacity to generate MDVs and are highly vulnerable to mild oxidative stress. Edward A. Fon's team conducted research demonstrating the involvement of the SNARE protein STX17 in the transportation of MDVs to lysosomes²²⁷. Building upon these findings, our previous studies have also confirmed that STX17-positive MDVs undergo rapid degradation in response to antimycin A, which can be effectively inhibited by lysosome inhibitors⁴¹¹. It has been well-established that oxidative stress plays a significant role in mitochondrial dysfunction during the aging process⁴⁶⁶. With advancing age, the accumulation of mutations and oxidative damage leads to a decline in mitochondrial DNA volume, integrity, and overall function⁴⁶⁷. Elderly individuals exhibit several characteristic features in their mitochondria, including reduced oxidative capacity, decreased oxidative phosphorylation, impaired ATP synthesis, elevated production of ROS, and compromised antioxidant defense

mechanisms ²⁷⁸. Furthermore, age-related alterations in mitochondrial dynamics and the suppression of mitophagy, an autophagic process responsible for the removal of damaged mitochondria, contribute to a decline in mitochondrial biogenesis ⁴⁶⁸. As a result, age-dependent abnormalities in mitochondrial quality control weaken and impair mitochondrial function. Assessing mitochondrial quality during the aging process can potentially serve as an early indicator of aging. However, effectively modulating the dynamics of mitochondrial quality remains a significant challenge. In our *in vivo* study, MDVs were isolated from the livers of young and middle-aged hSOD1^{G93A} and hSOD1^{WT} transgenic mice. Interestingly, we observed that MDVs derived from the middle-aged mice from both groups contained a substantial amount of oxidized hSOD1. This finding suggests that the formation and release of MDVs may play a role in the clearance of oxidized hSOD1 and contribute to mitochondrial quality control mechanisms in the context of aging. Specific mitochondrial compartments were utilized to quantify the production of MDVs. The single-membrane MDVs originating from the OMM were found to recruit all β -Barrel proteins, as well as the mitochondrial-anchored protein ligase (MAPL or Mul1) and the outer membrane translocase 20 (TOM20) ²³⁶. The TOM complex, known as the "entry gate," facilitates the import of precursor proteins synthesized by cytosolic ribosomes into the mitochondria. On the other hand, the double-membrane MDVs are generated from both the OMM and the IMM, occasionally containing matrix proteins ⁴⁶⁹. These MDVs are especially enriched with components such as the OXPHOS complexes, including complexes III and V, as well as the Fe-S cluster, which helps prevent excess mitochondrial iron and eliminate irreversibly damaged proteins ⁴⁷⁰. Additionally, MDVs may contain various proteins from the mitochondrial matrix, including pyruvate dehydrogenase, enzymes involved in the TCA cycle, fatty acid β -oxidation, and the antioxidant enzyme SOD2 ²²⁹. Furthermore, it has been observed that MDVs can also transfer

mitochondrial DNA (mtDNA), a mechanism potentially linked to systemic inflammation in conditions such as Parkinson's disease ²¹⁵. Notably, another group of MDVs has been identified to carry both the OMM-related protein TOM20 and the matrix-localized SOD2 ²³⁶. These findings highlight the diverse composition and potential functional roles of MDVs in maintaining mitochondrial homeostasis. However, the yield of various types of MDVs remains unclear. In our study, we employed specific mitochondrial compartments, PDH and TOM20, to assess the yield of MDVs isolated from the livers of mice belonging to different age groups. The production of PDH⁺ MDVs or TOM20⁺ MDVs was observed to be significantly higher in the aged groups (135 days-G93A, 17 months-WT) compared to the young groups (50 days-G93A, 135 days-WT). This suggests that the hSOD1 mutation greatly accelerated the aging process, and the generation of MDVs could serve as a novel indicator of aging.

Previous studies have demonstrated that MDVs can be detected under physiological conditions and can be further enhanced in the presence of mild oxidative stress without depolarization ²²⁹. Various types of stress have been reported to induce the formation of MDVs, including antimycin A, xanthine/xanthine oxidase, doxorubicin, glucose oxidase, and heat stress, among others. Among these, antimycin A, a complex III inhibitor, has been frequently used as an activator of MDV formation in most studies ⁴⁴⁵. MDVs are considered an early response to oxidative stress and play a role in maintaining mitochondrial homeostasis. Our previous investigations have shown that both OPCs and CHO cells can generate MDVs, and the extent of MDV production is dependent on the duration or intensity of the oxidative stress applied ⁴¹¹. If cellular stress persists for an extended period or reaches a high severity, mitochondria may undergo fragmentation, resulting in a reduction in MDV formation. Therefore, the responsiveness of mitochondria to antimycin A can serve as an indicator of mitochondrial quality. To measure the yield of MDVs, we isolated

mitochondria from mouse livers, which contain a significant number of mitochondria, and subsequently induced MDV formation by incubating the isolated mitochondria with AA for 2 hours. Our findings indicate that, even in the absence of the antimycin A, middle-aged mitochondria exhibited a higher MDV yield compared to young mitochondria, despite lacking morphological differences. This suggests that a higher level of oxidized proteins in middle age promotes MDV formation. Additionally, the accumulation of oxidized SOD1 contributes to reduced mitochondrial responsiveness, as evidenced by the decreased yield of MDVs in response to the challenge of AA. Consequently, a decline in mitochondrial responsiveness could serve as an early indicator of aging. In other words, MDVs offer a novel approach to assessing mitochondrial quality by collecting and quantifying their production.

In summary, understanding and controlling the intricate mechanisms that regulate mitochondrial quality control is essential for developing interventions to improve mitochondrial function and counteract age-related deterioration. While significant progress has been made in unraveling the role of MDVs involved in mitochondrial quality control, translating this knowledge into effective strategies to modulate mitochondrial dynamics and maintain mitochondrial health is still a complex task. Continued research and exploration are necessary to uncover novel approaches and therapies that can effectively enhance mitochondrial quality and preserve overall health and longevity.

Part IV Limitations

Although our research presents some innovative and encouraging findings, it does possess certain limitations that warrant acknowledgment.

Proteins are primary targets for oxidation due to their large abundance in cells, extracellular tissues, and bodily fluids, as well as their quick reaction rates with oxidants. Moreover, oxidative stress can break lipids and carbohydrates into highly reactive intermediates that target proteins at multiple functional locations. As a result, protein oxidation, glycooxidation, and lipoxidation produce a wide range of unique posttranslational protein changes. Irreversible alterations may lead to adverse circumstances and disorders, but reversible modifications are crucial in physiological processes and represent signalling systems ("redox signalling"). A growing body of research shows that they have a role in the start and progression of diseases and the aging process. Some protein oxidation products are chemically stable and abundant, making them potential candidates for oxidative damage indicators.

In our lab, we assessed multiple approaches for detecting the level of oxidized hSOD1. For example, the MalPEG modification was utilized to examine thiol group oxidation in hSOD1. The results indicate that the oxidation of cysteine 111 in SOD1 increased with age and was significantly higher in the aged group than the younger group⁴⁷¹. However, beyond the cysteine residues of hSOD1 being oxidized, there are other forms of oxidative modifications, such as nitration, acetylation, and phosphorylation⁴⁷². Therefore, MalPEG modification may not exclusively target the intended thiol groups and can react with other nucleophilic residues or molecules in the sample, resulting in non-specific modifications. Besides, achieving complete modification of all thiol groups in the target protein can be challenging. Furthermore, the modification process itself can potentially alter the native conformation and activity of the protein. This interference may introduce artifacts and affect the physiological relevance of the observed modifications. In our

study, we applied ELISA to test the level of the thiol group of hSOD1 in total SOD1. We assumed that the more oxidatively modified hSOD1, the less reactive thiol group of hSOD1 could be tested. For the ELISA assay, samples were incubated for one day in an aerobic chamber at room temperature to enable cysteine oxidation, whereas anaerobic preserved samples served as a control. The capture antibody SOD1 was incubated in wells of an ELISA plate overnight. Then hSOD1-containing samples were added and incubated for several hours the next day. Activated HRP containing a maleimide group that may react with thiol containing SOD1 was subsequently utilized to evaluate the thiol content of the captured SOD1 between groups. The results show a lower thiol group of hSOD1, indicating higher oxidized hSOD1 levels in aged groups than younger groups. The ELISA assay is sensitive, rapid, and straightforward method, albeit indirect, for detecting oxidized hSOD1.

Additionally, our research encompasses a preliminary characterization of MDVs isolated from mouse liver. It is important to acknowledge that our investigation focused solely on specific components of mitochondrial proteins within the MDV cargo. To attain a comprehensive understanding of MDVs as exosomes and their connections, it would be beneficial to explore other biomolecules, such as mitochondrial DNA and enzymes. Moreover, in order to authenticate and enhance our discoveries, it is imperative to undertake a comprehensive examination of MDVs, encompassing their structure and contents, through the utilization of Immunogold electron microscopy. This would provide deeper insights into morphologic changes and molecular modulations of MDVs. Moreover, we assessed the mitochondrial response capability by evaluating the yield of MDVs derived from liver mitochondria in both young and middle-aged individuals. This approach serves as an indicator of mitochondrial quality. However, it is crucial to extend our investigations to include MDVs isolated from other tissues, such as the brain, spinal cord, skeletal

muscles, and others. Future studies should continue to evaluate MDVs to assess mitochondrial quality across different tissues. In summary, through the expansion of our research and the inclusion of additional analyses, we can acquire a more comprehensive understanding of MDVs, their role in mitochondrial function, and their potential implications in the aging process.

Part V

Conclusions and Future Directions

5.1 Conclusions

In this study, we demonstrated that oxidized SOD1 is a causal factor of cellular senescence. Targeting oxidized SOD1 delays cellular senescence. Impairment of mitochondrial quality control is a mechanism of aging. Mitochondrial responsiveness to produce MDVs is an indicator of early aging. Oxidized hSOD1 in MDVs is a potential therapeutic target for delaying aging.

5.2 Future directions

Studies on MDVs have recently concentrated on numerous tissues and cells to get a more profound knowledge of MDVs; nevertheless, the physiological importance of different cell types, particularly *in vivo*, is still not fully known. Several shared variables regulate MDVs and mitophagy. Parkin and STX17, for instance, have comparable functions in both routes. Significant distinctions are also seen depending on the stress level, PINK1-initiated MDVs or mitophagy. More research on MDVs is required to clarify how this pathway changes with aging and age-related diseases, including but not limited to finding novel regulators, trigger factors, and roles. Our understanding of the payloads integrated into these vesicles is now restricted to just a few previously discovered proteins using immunofluorescence methods since extracting MDVs from tissue or cultured cells is challenging. Future research should concentrate on the creation of MDV-specific markers, the production of fluorescent antibodies, or the use of CRISPR technology to fluorescently identify endogenous genes.

Recent studies show therapeutic interventions involving EV targeting oxidative hSOD1 have demonstrated promising effects in extending lifespan, rejuvenating senescent cells, and alleviating age-related diseases. Since MDVs may have dual roles in aging, whether oxidized SOD1 is delivered to lysosomes for degradation or released extracellular space has not been elucidated. Strategies for MDV-targeting therapeutics could also be a novel anti-aging direction. Given that

MDVs have been found in EVs ²³², MDV-rich EVs may also shed light on the biomarker and therapeutic strategies for aging. Utilizing the CT4 peptide to encapsulate MDVs represents a promising therapeutic approach to delay or reverse the aging process. Finally, identifying aging signaling pathways may also open a new avenue to developing promising targets for healthy aging.

Abbreviations

AA	Antimycin A
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATF	Activating transcription factor
BCL2	B-cell lymphoma 2
BNIP3	Bcl-2 interacting protein 3
BRG1	Brm/Swi2-related gene 1
CCS	Copper chaperone
CDKs	Cyclin-dependent kinases
CDKIs	CDK inhibitors
CHOP	C/EBP homologous protein
ClpP	Clp protease proteolytic subunit
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
COX17	Cytochrome c oxidase copper chaperone encoded by <i>COX17</i> gene
CR	Calorie restriction
CSF	Cerebrospinal fluid
CTM	CMA targeting motif
DBR	Derlin-1 binding region of SOD1
DR	Dietary restriction

DRP1	Dynamin-related protein1
EM	Electronic microscopy
ER α	Estrogen receptor α
ETC	Electron transport chain
EVs	Extracellular vesicles
EVs ^{G93A}	EVs derived from NSCs ^{G93A}
EVs ^{WT}	EVs derived from NSCs ^{WT}
FOXO3	Forkhead box O3
FUNDC1	FUN14 domain-containing 1
GSK-3 β	Glycogen synthase kinase-3 β
GST	Glutathione s-transferase
GTP	Guanosine triphosphate
HMGB1	High mobility group box 1
HSF1	Heat shock factor 1
hSOD1	Human superoxide dismutase 1
hSOD1 ^{G93A}	G93A mutation of hSOD1
hSOD1 ^{WT}	Wild-type human SOD1
HSPs	Heat shock proteins
HTRA2	HtrA Serine Peptidase 2
IMM	Inner mitochondrial membrane
IMS	Inner membrane space
LAMP1	Lysosomal-associated membrane protein 1
LC3	Light chain 3

LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MAPL	Mitochondrial-anchored protein ligase
MDA	Malondialdehyde
MDVs	Mitochondrial-derived vesicles
MFN	Mitofusin
MND	Motor neuron disease
MIRO1/2	Mitochondrial Rho GTPase 1
MQC	Mitochondrial quality control
mtDNA	Mitochondrial DNA
mtHSPs	Mitochondrial heat shock proteins
NAD ⁺	Nicotinamide Adenine Dinucleotide
NDP52	Nuclear dot protein 52 kDa
NIX	BNIP3L
NRF1/2	Nuclear respiratory factor 1/2
NRs	Nuclear receptors
NSC	Neural stem cell
NSCs ^{-/-}	Non-transgenic NSCs
NSCs ^{G93A}	NSCs expressing the G93A mutation of hSOD1
NSCs ^{WT}	NSCs expressing the wild-type hSOD1
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
OPCs	Oligodendrocyte progenitor cells

OPTN	Optineurin
P1	Passage 1
PBST	PBS containing 0.25% Triton X-100
PD	Parkinson disease
PDH	Pyruvate dehydrogenase protein
PGC-1 α	Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha
PINK1	Phosphatase and tensin homolog induced putative kinase 1
PKB	Protein kinase B
PPAR α	Peroxisome proliferator-activated receptor alpha
PTMs	Post-translational modifications
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
SA- β -gal	Senescence-associated beta-galactosidase
SASP	Senescence-associated secretory phenotype
SEM	Standard error of the mean
SIRT1	Sirtuin1
SNAP29	SNARE-binding protein synaptosome-associated protein of 29 kDa
SNX9	Sorting Nexin 9
SOD1	Superoxide dismutase 1
SSBP1	Single-stranded DNA binding protein 1
STX17	Syntaxin 17
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy

TFAM	Mitochondrial transcription factor A
TFEB	transcription factor EB
TFs	Transcription factors
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane
UPRmt	Mitochondrial unfolded protein response
VAMP7	Vesicle-associated membrane protein 7
VDAC	Voltage-gated ion channel

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Review

Mitochondrial dysfunction in aging

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Mitochondrial dysfunction

ABSTRACT

Aging is a complex process that features a functional decline in many organelles. Although mitochondrial dysfunction is suggested as one of the determining factors of aging, the role of mitochondrial quality control (MQC) in aging is still poorly understood. A growing body of evidence points out that reactive oxygen species (ROS) stimulates mitochondrial dynamic changes and accelerates the accumulation of oxidized by-products through mitochondrial proteases and mitochondrial unfolded protein response (UPR^{mt}). Mitochondrial-derived vesicles (MDVs) are the frontline of MQC to dispose of oxidized derivatives. Besides, mitophagy helps remove partially damaged mitochondria to ensure that mitochondria are healthy and functional. Although abundant interventions on MQC have been explored, over-activation or inhibition of any type of MQC may even accelerate abnormal energy metabolism and mitochondrial dysfunction-induced senescence. This review summarizes mechanisms essential for maintaining mitochondrial homeostasis and emphasizes that imbalanced MQC may accelerate cellular senescence and aging. Thus, appropriate interventions on MQC may delay the aging process and extend lifespan.

1. Introduction

Aging is a complex process that involves a decline in biological and metabolic activity, resulting in the onset of various age-related conditions, such as neurodegenerative diseases, cardiovascular diseases, metabolic diseases, immune system diseases, and cancer (Guo et al., 2022). The past two decades have witnessed a wealth of promising work on age-associated factors that help us better understand aging and uncover interventions that can increase human longevity and improve quality of life (Costa et al., 2019; Longo and Anderson, 2022; McConnel and Turner, 2005).

One key aspect of aging that has been extensively studied is its impact on mitochondrial function. Mitochondria facilitate the fundamental energy conversion processes within cells (Waltz and Giege, 2020). In addition, they are the primary intracellular source of reactive oxygen species (ROS) generated during oxidative phosphorylation (Nohl et al., 2005). It is now generally recognized that ROS plays crucial roles in cellular differentiation and homeostasis (Sena and Chandel, 2012). In

addition, each ROS has its own set of characteristics. Highly reactive species, such as hydroxyl radical or peroxynitrite, may initiate lipid peroxidation when they react with membrane lipids and destroy various kinds of biological molecules, resulting in more complicated radicals and severe oxidative stress; whereas less reactive species, including superoxide and hydrogen peroxide, contribute to cellular signaling (Auten. and Davis, 2009; Collin, 2019). Apart from participating in intrinsic cell signaling pathways, ROS contributes to processes associated with aging (Warraich et al., 2020). While increased levels of mitochondrial ROS may not be the direct cause of aging according to existing evidence (Anagnostou and Hepple, 2020; Santos et al., 2018; Sanz and Stefanatos, 2008; Zhang et al., 2022), they do contribute significantly to the development of age-related diseases when combined with other factors, such as epigenetic alterations or a decrease in quality control systems (Elfawy and Das, 2019; Singh et al., 2019; Zhu et al., 2022). On the other hand, mitochondrial activity may not decline linearly throughout the aging process (Baker and Peleg, 2017). Research suggests that middle-aged individuals experience increased mitochondrial ROS as a

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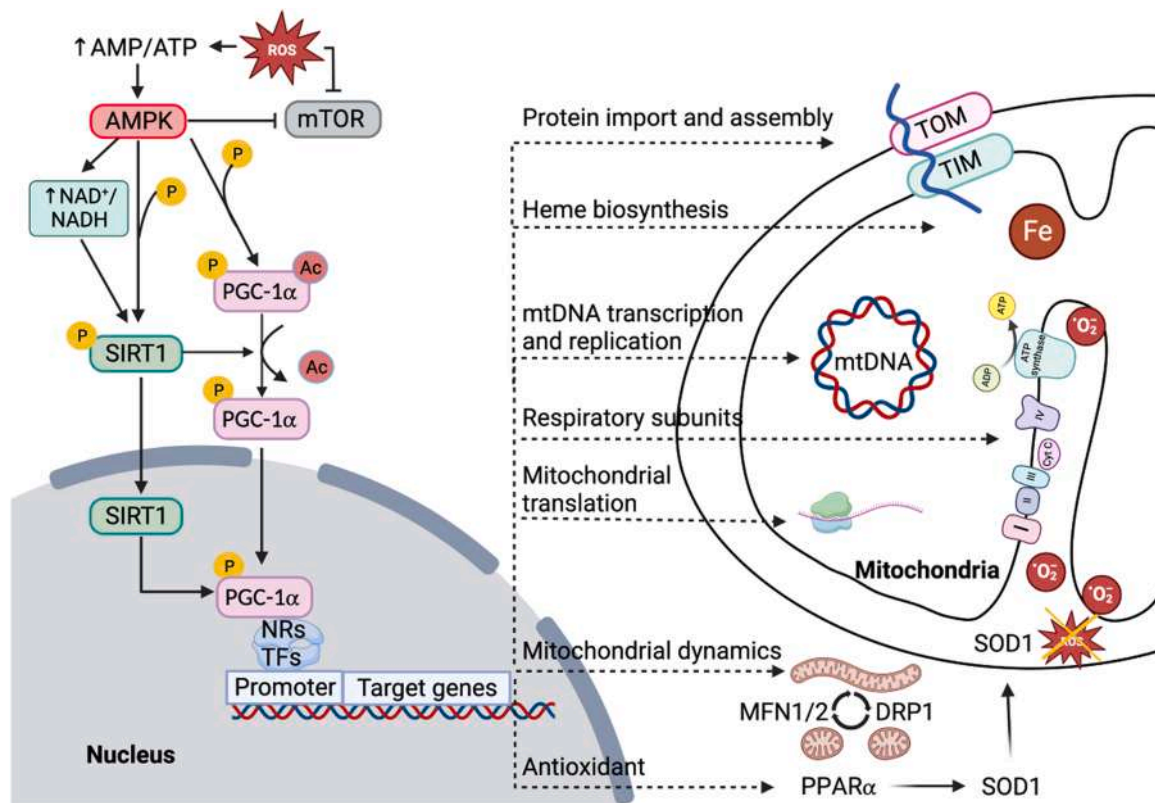


Fig. 1. Mitochondrial biogenesis and function. Mitochondrial biogenesis is a complex process involving several signaling pathways. AMPK is an energy sensor that detects the AMP/ATP ratio in response to mitochondrial ROS and is activated in low-energy settings. When AMPK is activated, it raises the NAD^+/NADH ratio and phosphorylates sirtuin 1 (SIRT1), which deacetylates PGC-1 α . It can also directly phosphorylate PGC-1 α while inhibiting mammalian target of rapamycin (mTOR) action. The activated PGC1 α co-activates NRs/TFs and regulates the nuclear target genes encoding proteins involved in mitochondrial biogenesis, such as protein import and assembly (TOM20, TOM70, COX17), respiratory components (complexes I, III, IV, and V), and heme biosynthetic enzymes in the mitochondrial matrix. There is also mounting evidence that target genes are not only responsible for regulating the expression of mitochondrial translational components such as ribosomal proteins and tRNA synthetases but also for mitochondrial DNA (mtDNA) transcription and replication. Furthermore, several proteins, such as mitofusin 1/2 (MFN1/2) and dynamin-related protein (DRP1), are synthesized to maintain mitochondrial dynamics. PPAR α activates the expression of copper-zinc superoxide dismutase 1 (SOD1) protein. AMPK, AMP-activated protein kinase; ROS, Reactive oxygen species; NAD^+/NADH , Nicotinamide Adenine Dinucleotide; SIRT1, Sirtuin1; PGC-1 α , Peroxisome proliferator-activated receptor-gamma coactivator-1 α ; FOXO3, Forkhead box O3; NRs, Nuclear receptors; TFs, Transcription factors; NRF1/2, Nuclear respiratory factor 1/2; COX17, cytochrome c oxidase copper chaperone encoded by COX17 gene; TOM/TIM complex, translocase of the outer/inner mitochondrial membrane complex; TFAM, mitochondrial transcription factor A; MFN1/2, mitofusin 1/2; DRP1, dynamin-related protein1; PPAR α , peroxisome proliferator-activated receptor alpha; SOD1, superoxide dismutase 1; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; GSK-3 β , glycogen synthase kinase-3 β .

signal to activate protective systems (Santos et al., 2021). However, persistent induction of ROS can lead to oxidative damage in old age (Barbieri and Sestili, 2012). As a result, ROS has the potential to accelerate the onset of aging and diminishes the quality of life in senior individuals (Singh et al., 2019).

Mitochondrial quality control (MQC) keeps ROS relatively low and steady (Figge et al., 2013). It disposes of the damaged part of mitochondria through mitochondrial proteases, mitochondrial unfolded protein response (UPR^{mt}), mitochondrial-derived vesicles (MDVs), and mitochondrial autophagy/mitophagy. Mitochondrial proteases perform numerous proteolytic tasks through MQC, including removal of damaged or misfolded polypeptides, regulation of protein processing, maturation cleavage, and assembling (Levytsky et al., 2016). UPR^{mt} enhances misfolded protein refolding and degradation in mitochondria (Zhu et al., 2021). Meanwhile, MDVs and mitophagy partially or entirely dispose of the damaged mitochondria and oxidized by-products (Picca et al., 2022). A complex signaling network is involved in the above mitochondrial quality control (Naresh and Haynes, 2019).

Aging causes a decrease in the efficiency of oxidative phosphorylation, leading to alterations in ATP production and an increase in ROS production (Macedo et al., 2021). This increased ROS production can lead to the oxidization of mitochondrial DNA, proteins, and lipids, which further reduces mitochondrial dynamics and impedes mitophagy,

ultimately resulting in mitochondrial dysfunction (Bakula and Scheibye-Knudsen, 2020; Juan et al., 2021). This creates a vicious cycle where dysfunctional mitochondria generate more ROS, worsening mitochondrial damage and reducing biogenesis further (Payne and Chinnery, 2015). While this cycle is closely related to the aging process, a thorough understanding of the mechanisms underlying MQC may help identify potential therapeutic targets to extend the overall healthy lifespan (Giorgi et al., 2018).

2. Mitochondrial function and biogenesis

Mitochondria are membrane-bound organelles, constituting one-fifth of the cellular volume in most eukaryotic cells. Each mitochondrion has outer and inner membranes. Large molecules and ions are primarily permeable to the outer mitochondrial membrane (OMM) but hardly permeable to the inner mitochondrial membrane (IMM) (Moriciano et al., 2021). Mitochondria are crucial in cellular metabolism and responsible for producing ATP. An electrochemical potential gradient across the IMM harbours the electron transport chain (ETC) to synthesize ATP through oxidative phosphorylation. Apart from energy metabolism, mitochondria are also responsible for other activities, such as heat generation, calcium and iron exchange, heme biosynthesis, cellular differentiation, proliferation, apoptosis, and cell death (Kluge et al.,

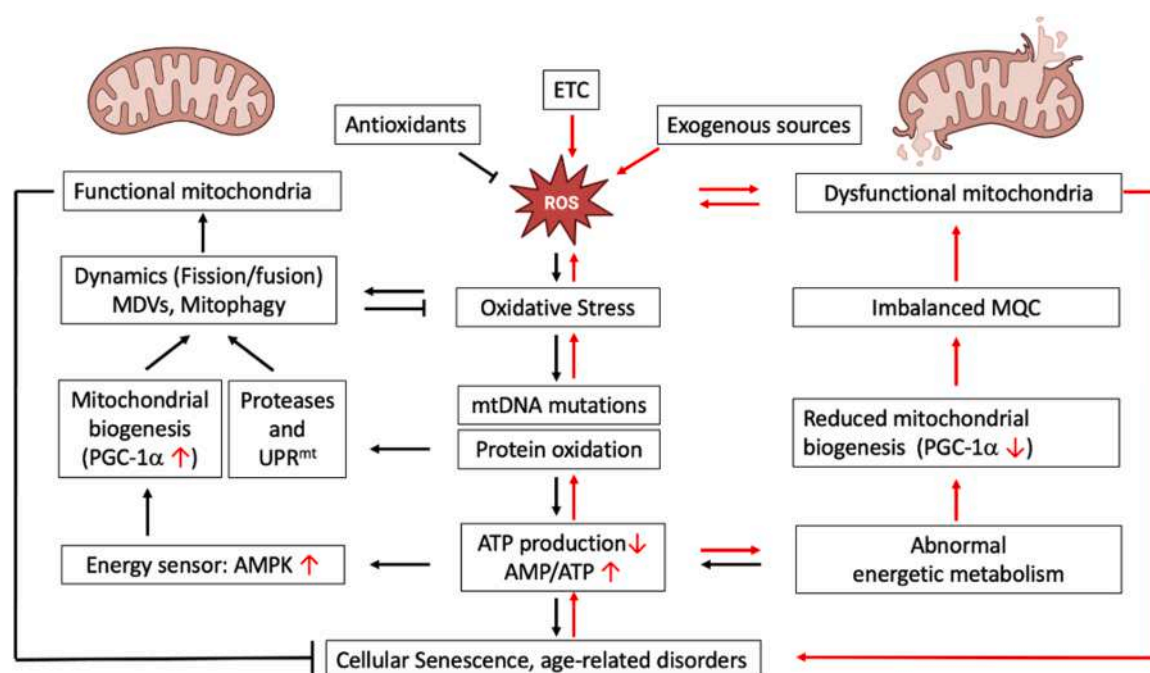


Fig. 2. Mitochondrial dysfunction in aging. ROS is generated from mitochondrial ETC and exogenous sources. Enzymatic and non-enzymatic antioxidants can convert ROS into less harmful by-products in physiological conditions. Excessive ROS induces oxidative stress, which leads to mitochondrial DNA mutations and protein oxidation. The abnormal products in mitochondria reduce ATP production and increase AMP/ATP ratio. AMPK, an energy sensor, could upregulate mitochondrial biogenesis and promote quality control. However, persistently decreased production of ATP induces abnormal energetic metabolism, which causes reduced mitochondrial biogenesis, imbalanced MQC, and mitochondrial dysfunction. These aberrant changes ultimately lead to cellular senescence and age-related disorders. ETC, electron transport chain; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; MQC, mitochondrial quality control; PGC-1 α , Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha; UPR^{mt}, Mitochondrial unfolded protein response.

2013).

Mitochondrial biogenesis takes place in healthy cells with a complex and multistep process. Induction of mitochondrial biogenesis involves activating transcription factors that regulate mitochondrial genes through cooperation between the nucleus and mitochondria (Popov, 2020). One essential step in mitochondrial biogenesis is the activation of transcription factors that regulate mitochondrial genes. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) is the master regulator. PGC-1 α is activated in response to changes in metabolic state. AMP-activated protein kinase (AMPK) is an energy sensor that detects the AMP/ATP ratio in response to mitochondrial ROS and is activated in low-energy settings. When AMPK is activated, it directly or indirectly phosphorylates PGC-1 α . The latter pathway is activated via raising the Nicotinamide Adenine Dinucleotide (NAD)--dependent deacetylase sirtuin 1 (SIRT1) (Kong et al., 2022). PGC-1 α modulates mitochondrial biogenesis by co-activating nuclear receptors and transcription factors. Once activated, the downstream genes involved in mitochondrial biogenesis are triggered, such as protein import and assembly (TOM20, TOM70, COX17) (Mercy et al., 2005), respiratory components (complexes I, III, IV, and V) (Rona-Voros and Weydt, 2010), and heme biosynthetic enzymes in the mitochondrial matrix (Handschin et al., 2005). There is also mounting evidence that target genes are not only responsible for regulating the expression of mitochondrial translational components such as ribosomal proteins and synthetases but also for mitochondrial DNA (mtDNA) transcription and replication (Kang, Li, 2012; Scarpulla, 2008). Furthermore, several proteins, such as mitofusin 1/2 (MFN1/2) and dynamin-related protein (DRP1), are synthesized to maintain mitochondrial dynamics (Halling and Pilegaard, 2020). PPAR α activates the expression of copper-zinc superoxide dismutase 1 (SOD1) protein (Wang et al., 2021). Thus, the number and functionality of mitochondria are ensured to meet the cellular energy demand (Fig. 1).

3. Mitochondrial dysfunction in aging

Aging is a chronic functional decline with multiple biological disarrangements. Mitochondrial dysfunction is one of the hallmarks of aging (Hernandez-Segura et al., 2018) and is related to many age-associated diseases, such as Cockayne syndrome (Fang et al., 2014), ataxia telangiectasia (Fang et al., 2016), xeroderma pigmentosum (Scheibye-Knudsen et al., 2014), Werner syndrome (Fang et al., 2019a), Parkinson's disease (PD) (Malpartida et al., 2021), Alzheimer's disease (AD) (Perez Ortiz and Swerdlow, 2019), and amyotrophic lateral sclerosis (Dafinca et al., 2021). Several factors are demonstrated to trigger the decline of mitochondrial function during aging, including elevated ROS production, mitochondrial DNA (mtDNA) mutations, protein oxidations, abnormal energy metabolism, and reduced mitochondrial biogenesis (Amorim et al., 2022; Natarajan et al., 2020) (Fig. 2).

3.1. Elevated ROS production and oxidative stress in aging

As organisms age, there is a gradual decline in physiological functions, which is accompanied by increased production of ROS and oxidative stress (Di Meo et al., 2016). ROS are highly reactive molecules that are generated as a by-product of normal metabolic processes, such as cellular respiration, and can damage cellular components such as proteins, lipids, and nucleotides (Juan et al., 2021). ROS production is triggered by various exogenous factors including oxidants in foods and nutrients and endogenous oxidative reactions such as respiratory chain reactions, lipid peroxidation, and mitochondrial oxidative phosphorylation (Zhang et al., 2021). ROS includes superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen, and alkoxyl and peroxy radicals (Singh et al., 2022b). The production of ROS is a natural consequence of various cellular processes, and the body has developed several mechanisms to counteract their damaging effects.

As we age, our body becomes less efficient in neutralizing ROS and

accumulates oxidative damage (Stefanatos and Sanz, 2018). The constant production of ROS may modify enzyme activities and change biomolecular structures (Checa and Aran, 2020). Enzymatic antioxidants, such as SOD1, glutathione peroxidase, glutathione-S-transferase, and catalase, convert ROS into less harmful by-products in physiological conditions (Babiuch et al., 2019). Imbalanced generation and neutralization of ROS accelerate oxidative stress; vice versa, oxidative stress can also induce ROS generation (Wei and Lee, 2002). Oxidative stress has been linked to a variety of age-related diseases, including neurodegenerative disorders, cardiovascular diseases, and cancers (Luo et al., 2020). It is thought that oxidative stress contributes to the pathogenesis of these diseases by promoting inflammation, cellular senescence, and DNA damage (Zhu et al., 2014).

Mitochondria are the primary site for ROS production in a cell; as such, changes in mitochondrial function can have a significant impact on oxidative stress (Chen et al., 2003). With age, there is a decline in mitochondrial function, leading to increased ROS production. Respiratory chain complexes I and III participate in the ubiquinone cycle in stimulating univalent oxygen reduction; complex II is responsible for superoxide anion production (Giorgi et al., 2018). In the aging process, excessive ROS continually accumulate in mitochondria, causing decreased ATP production and disrupted respiratory chain activity, ultimately leading to mitochondrial dysfunction and damage (Zorov et al., 2014). In neurons, for example, high levels of mitochondrial ROS can induce neuroinflammation and neuronal death (Stefanatos and Sanz, 2018).

3.2. Higher level of mitochondrial DNA mutations in aging

While aging is a natural process that occurs in all living organisms, a combination of genetic, lifestyle, and environmental factors might cause alterations in aging process. This premature aging can significantly impact on an individual's quality of life and lifespan (Uwineza et al., 2019). One contributing factor is genetic mutations that affect mitochondrial function. Specifically, mutations in mtDNA can impair energy production and increase the production of ROS, further exacerbating the aging process (Nissanka and Moraes, 2018). mtDNA consists of small circular chromosomes in mitochondria. It contains 37 genes that encode 13 proteins, 22 tRNAs, and 2 rRNAs (Taanman, 1999). mtDNA is hereditary and constantly affected by both exogenous and endogenous stresses. Besides, mtDNA is more vulnerable to mutations (approximately 10-fold higher) than nuclear DNA. Base excision repair, direct reversal, mismatch repair, and possibly double-strand break repair are proven to protect mtDNA. However, the genomes are very close to the respiratory complexes in the IMM, where ROS are generated in large quantity during oxidative phosphorylation (Wolf, 2021). Although the repair systems exist in mitochondria, they are still relatively limited to counteract the mutations without the protection of histone (Alexeyev, 2009). Consequently, mtDNA mutations can cause imbalanced mitochondrial respiration and accelerating ROS accumulation. This vicious circle contributes to cellular senescence and age-related disorders (Medala et al., 2021). A study shows that transgenic mice with mtDNA mutations display early aging phenotypes, such as hunchback, alopecia, grey hair, hearing loss, and weight loss, with shortened lifespans (Tadakuma et al., 2005). Aging humans also exhibit higher levels of mutations in mitochondrial genomes than young individuals in their brains, hearts, and skeletal muscles (Corral-Debrinski et al., 1992; Fivenson et al., 2017; Hiona and Leeuwenburgh, 2008). Further research in this field may provide some mechanistic understanding of aging and age-related neurological disorders (Hou et al., 2019).

3.3. Accumulation of oxidized proteins in aging

Proteins are a primary target of oxidative modifications during aging. Since mitochondria are a primary site for ROS production, mitochondrial proteins are particularly susceptible to oxidative

modifications (Ugarte et al., 2010). Thirteen mitochondrial gene-encoded proteins are synthesized in the mitochondrial matrix. In comparison, more than 1200 nuclear-encoded mitochondrial proteins are synthesized in the cytosol and delivered to the OMM, IMM, IMS, and mitochondrial matrix. The oxidative stress causes a plethora of post-translational protein modifications by targeting sulfur-containing moieties, such as cysteine and methionine. Protein cysteine residues are essential for stabilizing protein structures but are particularly vulnerable to oxidative modification, because their sulfhydryl moiety is easily attacked by redox-active substrates (Nagano and Araki, 2016). The first reaction by-product of a thiolate with H_2O_2 is sulfenic acid, which can then participate in various reactions depending on the circumstances. The sulfenic can form intermolecular or intramolecular disulfide bonds and be reversed by glutathione. Moreover, sulfenic can be oxidized to sulfinic and sulfonic acids. This sulfonic acid is the most highly oxidized thiol species, and its formation is irreversible (Reeg and Grune, 2015). MDVs and mitophagy could degrade the oxidatively modified proteins (Ng et al., 2021). However, excessively oxidized proteins in mitochondria deteriorate the capability of MQC (Yan et al., 2020). The misfolded aggregates of proteins, such as α -synuclein, amyloid-beta ($A\beta$), Tau, 43kda TAR DNA binding protein, or SOD1, have been reported to accumulate in the space of mitochondria that leads to mitochondrial dysfunction and neural degeneration (Jara et al., 2020; Lautenschlager et al., 2020; Mendez-Lopez et al., 2021; Wang et al., 2019c).

3.4. Decline of mitochondrial respiratory capacity and ATP production in aging

Mitochondrial health is essential for physiologic function, especially in tissues with a high ATP turnover rate. However, mitochondrial respiratory capacity and coupling quality control decline with age (Porter et al., 2015). The respiratory chain complexes (complexes I, II, III, and IV) and ATP synthase-complex V exist in the inner mitochondrial membrane and participate in oxidative phosphorylation. Electrons are transferred through the respiratory complexes to form ATP. In case of a sudden increase in energy demand, respiratory reserve capacity can be used to produce extra ATP through oxidative phosphorylation (Nolfi-Donagan et al., 2020). However, if the respiratory reserve capacities of mitochondria are exhausted, the affected cells undergo senescence or die (Ho et al., 2021). Studies have shown that pharmacological inhibition or genetic manipulation of mitochondrial respiratory complexes can induce cellular senescence (Atamna et al., 2012). Rotenone, a complex I inhibitor, upregulates senescence-associated β -galactosidase activity and ROS levels leading to cellular senescence (Ho et al., 2021). Deferoxamine mesylate is an FDA-approved iron chelator. It inhibits complex II activity and could either promote or prevent cellular senescence depending on its concentration and mode of application (Lee et al., 2021; Shatrova et al., 2021). Silencing of Rieske iron-sulfur polypeptide, a critical protein subunit of complex III, significantly inhibits ATP synthesis and leads to cellular senescence (Shi et al., 2018). Reduced levels of mitochondrial Coenzyme Q and decreased ETC efficiency are associated with age-related declines in mitochondrial function (Takahashi and Takahashi, 2019).

3.5. Abnormal AMPK-mediated energy metabolism in aging

There is growing evidence suggesting a decline in energy metabolism during aging. This process is closely connected to AMPK's positive activation and decreased ATP production in response to oxidative stress (Błaszczuk, 2020). AMPK triggers catabolism, such as glycolysis and fatty acid oxidation, while inhibits anabolism, such as protein synthesis (Flores et al., 2022). AMPK has also been proven to be a critical modulator of aging by inhibition of mammalian target of rapamycin (mTOR) and phosphorylation of SIRT1 (Giovannini and Bianchi, 2017). mTOR is a ubiquitously expressed serine/threonine-protein kinase within the PI3K family that controls cellular energy levels, as well as

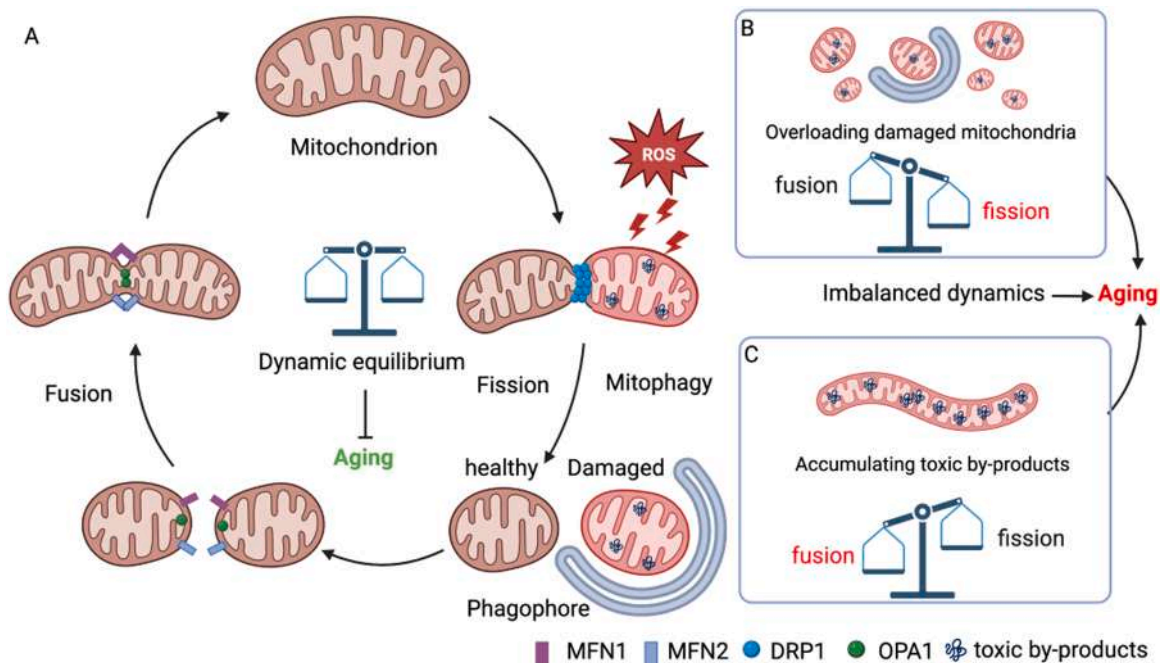


Fig. 3. Mitochondrial dynamics and aging. (A) Mitochondrial dynamic equilibrium is essential for mitochondrial homeostasis as they regulate the mitochondrial number, shape, function, and subcellular distribution. Many GTPases are involved in the regulation of mitochondrial dynamics. The DRP1 protein directs fission in mitochondria, and MFN1 and MFN2 on the outer membrane and OPA1 on the inner membrane mediate fusion. The damaged part of mitochondria is separated by DRP1 from the healthy part and is engulfed by a phagophore to undergo mitophagy. In addition, the healthy part of the mitochondria fuses with other mitochondria to fulfil its function for anti-aging. (B) Insufficient fusion or additional fission may lead to mitochondrial autophagy or fragmentation. (C) A deficiency of fission or excessive fusion often leads to a highly connected and extended mitochondrial network that may promote mitochondrial biogenesis but accumulate toxic by-products. Imbalanced dynamics may accelerate aging. GTP, guanosine triphosphate; DRP1, dynamin-related protein 1; MFN1/2, mitofusin 1 /2; OPA1, optic atrophy 1.

nutritional and growth factor status (Baretic and Williams, 2014). It has been demonstrated that deleting the mTORC1 component raptor in mouse skeletal muscle induces mitochondrial dysfunction (Bentzinger et al., 2008). SIRT1 deacetylates target substrates in an NAD^+ -dependent manner. Its activity directly correlates metabolic perturbations with transcriptional outputs. Moreover, SIRT1 substrates may extend beyond transcriptional regulators to directly deacetylate the acetyl-CoA synthetase and mitochondrial biogenesis-related proteins (Canto and Auwerx, 2009; Rabinovitch et al., 2017). AMPK signaling becomes less responsive as people age (Tadakuma et al., 2005). When AMPK activation is insensitive to cellular stress, such as in conditions like obesity or insulin resistance, energy metabolism is abnormal. Therefore, the abnormal energy metabolism caused by the insensitivity of AMPK activation can have significant consequences on cellular health and longevity, including imbalanced mitochondrial dynamics, reduced mitochondrial biogenesis, and, ultimately, mitochondrial dysfunction and cellular senescence (Wang et al., 2019a).

3.6. Reduced mitochondrial biogenesis in aging

Over the past decades, studies have shown that mitochondrial biogenesis decreases with age. PGC-1 α is the master regulator of mitochondrial biogenesis. Ample evidence supports the role of PGC-1 α in aging. Increased expression of PGC-1 α could compensate for the mitochondrial dysfunction and improve aging phenotypes in mtDNA mutation mice (Dillon et al., 2012). However, there is an inadequate response of PGC-1 α to exercise training in aged mice (Derbre et al., 2012). Mitochondrial transcription factor A (TFAM) is a downstream transcription coactivator of PGC-1 α . It regulates mtDNA transcription and packages mtDNA into nucleoids (Chen et al., 2022). Loss of TFAM leads to less mtDNA and impairment of the electron transport chain (Kang et al., 2018). Therefore, decreased levels of PGC-1 α and TFAM are

closely associated with inadequate mitochondrial biogenesis and age. However, oncogene-induced senescence shows elevated levels of both PGC-1 α and TFAM. Thus, over activated mitochondrial biogenesis might also be detrimental to cells (Moiseeva et al., 2009).

4. Imbalanced mitochondrial quality control during aging

A series of MQC maintains mitochondrial function and homeostasis. As MQC operates through a complex network of pathways, disruptions at any level may influence the entire system (Picca et al., 2018). Accumulated dysfunctional mitochondria due to failure of MQC and the expansion of mitochondrial defects eventually lead to cell senescence and death (Larsen et al., 2018).

4.1. Mitochondrial proteolysis and aging

Mitochondrial proteolysis is the frontline of mitochondrial quality control (Ahola et al., 2019). Many proteases, such as the Lon protease, ATP-dependent Clp protease proteolytic subunit (ClpP), and membrane-bound proteases, maintain proteostasis in mitochondria, degrade misfolded proteins and import newly synthesized proteins. The ubiquitin-proteasome system directs misfolded proteins for proteasomal degradation before being imported into mitochondria. The Lon and ClpP proteases in the mitochondrial matrix are responsible for the proteolysis of damaged proteins. Age-dependent decline in mitochondrial proteolysis leads to the accumulation of misfolded proteins, which promotes cellular stress and may contribute to aging. A study on mouse skeletal muscle shows that the activity of Lon levels declines with age (Bota and Davies, 2016). Increasing chronic oxidative stress may exacerbate Lon's deleterious effects with age (Ngo and Davies, 2007). Besides, there is evidence that ClpP $^{-/-}$ mice exhibit severe growth retardation, decreased spontaneous activity, and reduced survival phenotypes as they age

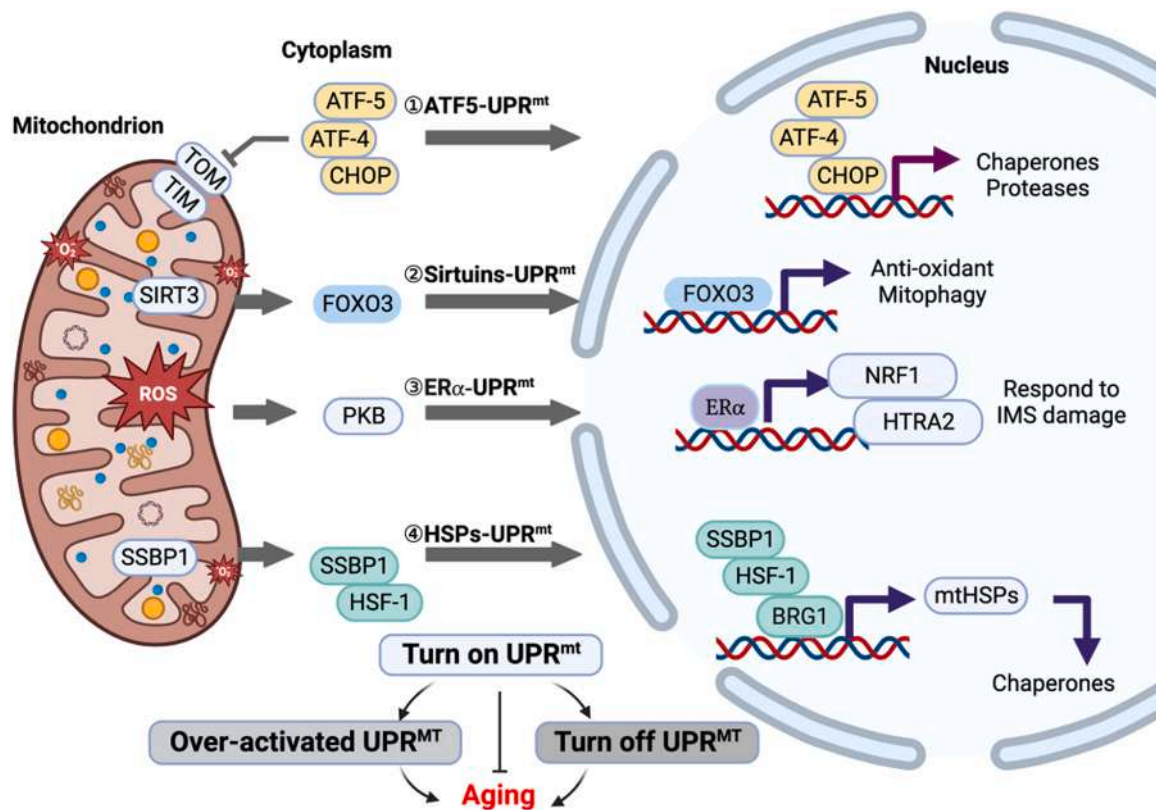


Fig. 4. Mitochondrial UPR^{mt} and aging. Oxidative stress activates four different axes of the UPR^{mt} in mammals, the ATF5-UPR^{mt} axis, Sirtuin-UPR^{mt} axis, ERα-UPR^{mt} axis, and HSPs-UPR^{mt} axis. ① Misfolded proteins in mitochondria activate the ATF5-UPR^{mt} axis, in which ATF5 conjugates with ATF4 and CHOP to translocate from mitochondria to the nucleus. The complex is an orthologous transcription factor of ATF5-1 in mammals that could upregulate genes involved in mitochondrial proteostasis. ② In response to oxidative stress, the SIRT3-UPR^{mt} axis activates Forkhead box O3 (FOXO3) to promote antioxidant stress and mitophagy, increasing the transcription of superoxide dismutase and catalase. ③ The kinase AKT activates the ERα-UPR^{mt} axis's estrogen receptors, increasing the HtrA Serine Peptidase 2 (HTRA2) production and nuclear respiratory factors (NRF1 and NRF2). ④ In the HSPs-UPR^{mt} axis, HSF1 interacts with Single-Stranded DNA Binding Protein 1 (SSBP1) to create a complex, which then attracts the chromatin-remodeling gene Brm/Swi2-related gene 1 (BRG1) and induces the production of mitochondrial heat shock proteins (mtHSPs). In summary, the mammalian UPR^{mt} promotes mitochondrial recovery and maintains proteostasis through various pathways. ATF, activating transcription factor; ERα, estrogen receptor α; HSPs, heat shock proteins; CHOP, C/EBP homologous protein; FOXO3, Forkhead box O3; PKB, protein kinase B; HTRA2, HtrA Serine Peptidase 2; NRF1/2, nuclear respiratory factor 1/2; HSF1, heat shock factor 1; SSBP1, single-stranded DNA binding protein 1; BRG1, Brm/Swi2-related gene 1; mtHSPs, mitochondrial heat shock proteins.

(Torres-Odio et al., 2021). Neurons with accumulation of tau proteins resulting from dysfunctional ubiquitin-proteasome systems show aberrant synaptic activities in AD patients (Upadhyaya and Hegde, 2007). As a result, impairment of this system could induce the accumulation of misfolded or damaged proteins, which may lead to aberrant cellular function and contribute to aging progression.

4.2. Mitochondrial dynamics and aging

Mitochondria are highly dynamic through fission and fusion. Fission breaks one mitochondrion into two, while fusion joins two mitochondria together. Fission and fusion dynamics help maintain mitochondrial number, morphology, functions, and subcellular distribution, which are essential to preserving mitochondrial health. GTPases of hydrolase enzymes control the mitochondrial network. The dynamic-related protein (DRP1) directs fission in mitochondria, while mitofusin 1/2 (MFN1 and MFN2) on the outer membrane and optic atrophy 1 (OPA1) on the inner membrane mediate mitochondrial fusion. A deficiency of fission or excessive fusion often leads to a highly connected and extended mitochondrial network (Rana et al., 2017; Yu et al., 2019), that may promote mitochondrial biogenesis but accumulate toxic by-products (Huang et al., 2007; Mopert et al., 2009; Wu et al., 2019). On the other hand, insufficient fusion or additional fission may lead to mitochondrial autophagy or fragmentation (Chun et al., 2018; Medala et al., 2021; Sebastián et al., 2016). Various senescent cells show enlarged cell bodies

with complicatedly interconnected and elongated mitochondria (Ren et al., 2020). Low expression of p-DRP1 and high expression of MFN2 in senescent mesenchymal stem cells has been found to promote mitochondrial fusion during aging (Hong et al., 2020). However, an opposite finding shows higher expression of DRP1 but lower expression of OPA1 in the brains of aged mice (Eckert et al., 2013) (Fig. 3). Hence, maintaining a balance between mitochondrial fission and fusion is crucial for preserving mitochondrial health and delaying aging.

4.3. Mitochondrial unfolded protein response (UPR^{mt}) and aging

UPR^{mt} is a molecular protective mechanism that helps refold misfolded and unfolded proteins in mitochondria to create a stable mitochondrial environment. UPR^{mt} retrogrades the stress signals from mitochondria to the nucleus (Fig. 4). In mammals, the accumulation of misfolded proteins in the intermembrane space and mitochondrial matrix can induce activating transcription factor 5 (ATF5) by conjugation with ATF4 and CHOP as a complex, which translocates from the cytosol into the nucleus. The complex is an orthologous transcription factor of ATF5-1 in mammals that could upregulate genes involved in mitochondrial ClpP and mitochondrial chaperone (Hsp60/10) gene expressions to enhance misfolded protein refolding and degradation (Lee et al., 2020; Zhu et al., 2021). Besides, the ATF5-UPR^{mt} axis, the activation of UPR^{mt} could also be activated through the HSPs-UPR^{mt} axis, sirtuin-UPR^{mt} axis, and ERα-UPR^{mt} axis in mammals. Studies have

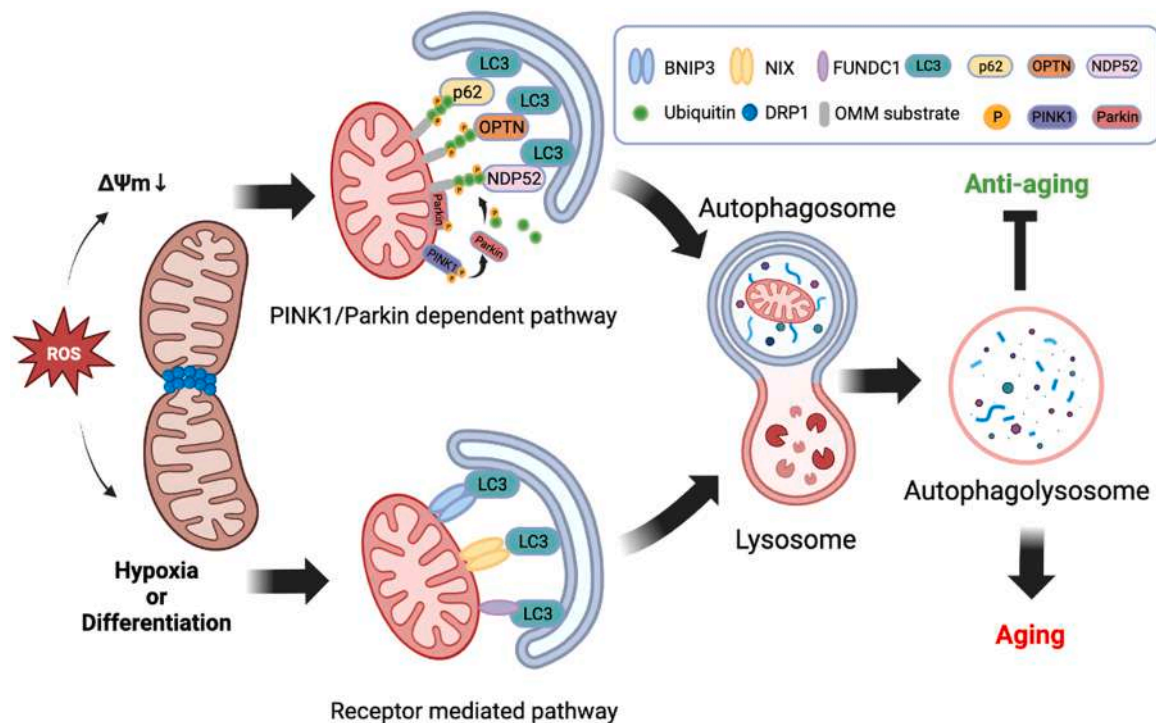


Fig. 5. Mitophagy and aging In healthy mitochondria, PINK1 can be eliminated constitutively after being imported to the inner membrane by the proteasome. When a mitochondrion undergoes oxidative stress, PINK1 import is impeded, and PINK1 accumulates on the outer membrane. Once docked on the outer membrane, PINK1 recruits and activates Parkin via phosphorylating S65 in Ubiquitin and Parkin's ubiquitin-like domain. Parkin then ubiquitinates mitochondrial substrates on the OMM, allowing LC3 attached to phagophores to be recruited via autophagy adaptors such as p62, OPTN, and NDP52. The phagophore swells and engulfs the mitochondrion, forming an autophagosome that merges with a lysosome to be degraded. Receptor-mediated mitophagy: The mitophagy receptor NIX, FUNDC1, and BNIP3 are strongly activated during reticulocyte maturation. This causes an increase in receptor levels on the OMM, where they can bind to LC3 conjugated to a phagophore through its LIR. After being engulfed by an autophagosome and fused with a lysosome, the mitochondrion is destroyed by lysosomal hydrolases. However, mitophagy induced by consistent accumulation of misfolded proteins and aggregates might be over-activated or even turned off in advancing age. Besides, insufficient mitophagy has also been found to induce mitochondrial dysfunction and cellular senescence. PINK1, phosphatase and tensin homolog induced putative kinase 1; OMM, outer mitochondrial membrane; LC3, light chain 3; OPTN, optineurin; NDP52, nuclear dot protein 52 kDa; BNIP3, Bcl-2 interacting protein 3; FUNDC1, FUN14 domain-containing 1.

demonstrated that the accumulation of misfolded proteins, such as APP/A β , tau, α -Synuclein, and SOD1^{G93A}, persists in activating UPR^{mt} in age-related neurodegenerative diseases (Hu et al., 2019; Poirier et al., 2019; Riar et al., 2017). However, UPR^{mt} may not repair severely damaged mitochondria with excessive misfolded proteins and aggregates, although it is highly activated (Schulz and Haynes, 2015). Intriguingly, histone-3 is abnormally trimethylated in senescent cells. This condition inhibits ATF-5 from binding with DNA, resulting in the shutdown of UPR^{mt} (Munoz-Carvajal and Sanhueza, 2020). Thus, activation of UPR^{mt} could be protective at an earlier stage of aging, but over-activated or turn-off UPR^{mt} may accelerate aging process (Merkwirth et al., 2016; Tian et al., 2016; Yoneda et al., 2004).

4.4. Mitophagy and aging

In recent years, many studies have focused on the role of mitophagy due to its close relationship with aging (Chen et al., 2020; De Gaetano et al., 2021). Mitophagy is a mitochondrial response to various stresses, including oxidative stress, nutrient starvation, or programmed removal of mitochondria (Bakula and Scheibye-Knudsen, 2020) (Fig. 5). Aging impairs mitochondrial function and mitophagy, which may compromise the integrity of the blood-brain barrier and diminish cerebrovascular health with aging (Tyrrell et al., 2020). Here we aim to summarize the underlying mechanisms between mitophagy and aging.

4.4.1. PINK1/Parkin-dependent mitophagy in aging

The phosphatase and tensin homolog-induced putative protein kinase 1 (PINK1) and E3 ubiquitin ligase Parkin are two significant factors

in the PINK1-Parkin pathway. In physiological conditions, PINK1 is imported into the mitochondrial matrix through translocase of the outer/inner mitochondrial membrane (TOM/TIM) complex and degraded by mitochondrial proteases. Under stressful situations, PINK1 is blocked on the depolarized OMM to recruit cytosolic ubiquitin and Parkin. PINK1 phosphorylates Parkin, causing Parkin's E3 ligase function to be activated. The activated Parkin is then recruited to the depolarized mitochondria and ubiquitinates outer mitochondrial substrates (Pickles et al., 2018). Although it's still unclear how these autophagy components are recruited to separated membranes forming around mitochondria, the adaptor proteins, including p62, Optineurin, and nuclear dot protein, bind to both ubiquitin chains and microtubule-associated protein 1 A/1B-light chain 3 (LC3) /GABARAP family members. After that, the autophagosome is transported to lysosome for digestion (Pickrell and Youle, 2015). It has been demonstrated that PINK1-mediated mitophagy has a protective role against mitochondrial dysfunction (Wang et al., 2018b). Mutations in both PINK1 and Parkin have been discovered in PD (Enza et al., 2004; Kitada et al., 1998). Evidence shows that mitophagy is also impaired in the hippocampus of AD patients and in a mouse model of AD (Cai and Jeong, 2020; Xie et al., 2022). Mitophagy reduces insoluble A β and protects against cognitive impairment in an AD mouse model via microglial phagocytosis of extracellular A β plaques and inhibition of neuroinflammation (Fang et al., 2019b). Therefore, mitophagy might be used as a therapeutic intervention. However, recent studies reveal that mitophagy may accelerate aging by activating the PINK/Parkin pathway (Huang et al., 2020; Zha et al., 2017). The discrepancy may result from the extent of mitophagy under different situations. Mitophagy helps the

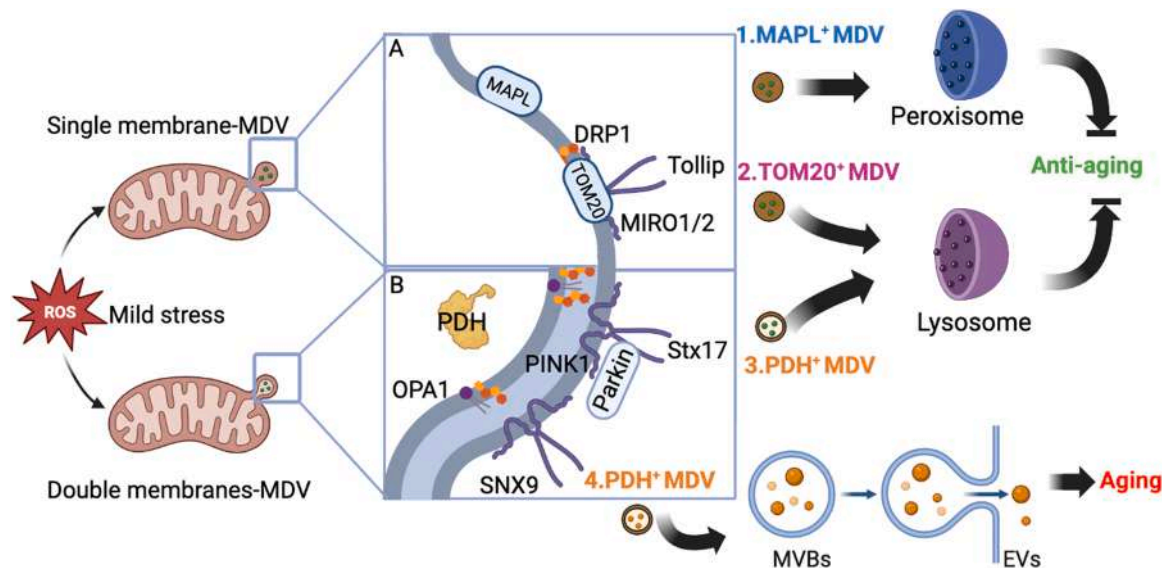


Fig. 6. MDVs and aging. Different molecular pathways and destinations may cause MDVs to delay or accelerate aging. MAPL⁺ MDVs with a single-layer membrane are directed to the peroxisome, while TOM20⁺ MDVs are initiated by MIRO1/2 and DRP1 and then transported to the lysosome by Tollip. Double-membrane PDH⁺ MDVs have two distinct destinations. PINK1/Parkin-dependent PDH⁺ MDVs are conveyed to lysosomes for degradation by a ternary SNARE complex (Syntaxin-17, SNAP29, and VAMP7). The oxidized by-products in the previously mentioned MDVs may be digested by lysosomes, which may assist mitochondria in staying healthy and delaying cellular senescence. When OPA1 and SNX9-directed PDH⁺ MDVs exceed the capacity of lysosomes, they are organized into multivesicular bodies (MVBs) and released as extracellular vehicles. The complex contents may be transported to neighbouring cells, thereby hastening cellular senescence. SNAP29: SNARE-binding synaptosome-associated protein of 29 kDa; VAMP7: vesicle-associated membrane protein 7; MIRO1/2, mitochondrial Rho GTPase 1; SNAP29, SNARE-binding protein synaptosome-associated protein of 29 kDa; VAMP7, vesicle-associated membrane protein 7; SNX9, Sorting Nexin 9, EVs, extracellular vesicles.

elimination of damaged mitochondria, but excessive mitophagy might impair mitochondrial function, which in turn accelerates aging (Yamamoto et al., 2020).

4.4.2. Receptor-mediated mitophagy

Receptor-mediated mitophagy can selectively remove damaged mitochondria through mitochondrial receptors. Both B-cell lymphoma-2 (BCL2) /adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and BNIP3L (NIX) belong to the BH3-only domain proteins of the BCL2 family. BNIP3 exists as a monomer in the cytosol but becomes a homodimer and recruits to OMM under hypoxia or differentiation stimulation (Onishi et al., 2021). Then the receptors interact with LC3 proteins localized in autophagosomes, which induce mitophagy (Poole et al., 2021). Another mitophagy receptor, NIX, also known as BNIP3L, has 53–56% amino acid sequence identity with BNIP3. It is dimerized under stress conditions, enabling mitochondria's engulfment by autophagosomes to initiate mitophagy (Rogov et al., 2017). The FUN14 domain containing 1 (FUNDC1) protein is another example of mitophagy receptor (Liu et al., 2012). In physiologic conditions, FUNDC1 undergoes phosphorylation by Src family kinase and casein kinase 2 proteins, resulting in reduced interaction with LC3 (Chai et al., 2021). Under stressful conditions, mitochondrial phosphoglycerate mutase 5 could dephosphorylate FUNDC1 to activate mitophagy (Yu et al., 2020). Studies have found NIX can induce mitophagy in compensating for mutations of PINK and Parkin (Koentjoro et al., 2017). BNIP3/NIX and FUNDC1 mitophagy may compensate for defective PINK1/Parkin-mediated mitophagy (Lampert et al., 2019). But lower mRNA levels of *Nix*, *bnip3*, *fundc1*, *Pink1*, *Parkin*, autophagy-related proteins 5 (*Atg5*), *Atg12*, and *Atg13*, lead to insufficient mitophagy and may lead to mitochondrial dysfunction and cellular senescence (Oh et al., 2020).

4.5. MDVs and aging

MDVs are mitochondrial membranous protrusions that act as novel mitochondrial quality control and may also play a vital role in health,

aging, and diseases (Picca et al., 2020b). Although MDVs are considered the piecemeal of mitophagy, they are distinct mitochondrial processes. Firstly, MDVs are generated from healthy mitochondria, whereas mitophagy occurs in depolarized ones (McLelland et al., 2014). Secondly, MDVs with single or double membranes have only 70–150 nm in diameter, which are much smaller than mitophagosomes (>300 nm in diameter) (Cadete et al., 2016). Thirdly, MDVs are independent of mitophagy-associated proteins, DRP1, ATG5, LC3, Beclin-1, or RAB9 (Chen et al., 2020; Quan et al., 2020; Soubannier et al., 2012). As a result, MDVs could compensate for the loss of mitophagy to maintain mitochondrial homeostasis (Towers et al., 2021). Seventy-two proteins are discovered in MDVs, including 31% oxidative phosphorylation proteins and highly enriched TIM chaperones, which indicates MDV transportation might be chaperone-mediated (Roberts et al., 2021). TOM20 and mitochondria-anchored protein ligase (MAPL) proteins are mitochondrial outer-membrane proteins. Pyruvate dehydrogenase (PDH) is the protein in the mitochondrial matrix. While the ultimate destiny of mitochondrial-derived vesicles (MDVs) remains uncertain, their selection is influenced by either their intended target or different conditions of mitochondrial stress (Konig et al., 2021; Neuspiel et al., 2008). MAPL⁺ MDVs are targeted at peroxisomes for degradation (Neuspiel et al., 2008). TOM20⁺ MDVs are associated with DRP1 and mitochondrial Rho GTPase 1/2 and will then be degraded within the lysosomes (Kaarniranta et al., 2018; Roberts et al., 2021; Soubannier et al., 2012; Todkar et al., 2021). On the other hand, a part of PDH⁺ MDVs shares the same PINK1/Parkin trafficking pathway with mitophagy (Ramirez et al., 2022). To facilitate their degradation, MDVs are transported to the lysosome through a ternary SNARE complex composed of syntaxin-17, SNARE-binding protein synaptosome-associated protein of 29 kDa (SNAP29), and vesicle-associated membrane protein 7 (VAMP7) (McLelland et al., 2016). In addition, Sorting nexin 9 (SNX9) is responsible for protein sorting and transport, vesicle formation, and endocytosis. SNX9 accumulates on the mitochondrial surface during mitochondrial antigen presentation to modulate the production of PDH⁺ MDVs (Matheoud et al., 2016). OPA1 and SNX9-directed PDH⁺ MDVs package

damage-associated molecular patterns into multivesicular bodies (MVBs) (Todkar et al., 2021; Vasam et al., 2021). Then MVBs fuse with cellular membranes and release MDVs to the extracellular space, stimulating mitochondria-driven immune responses (Fig. 6). Several pieces of evidence on the extracellular vesicles (EVs) are discovered to transport to the adjacent cells and proven to trigger innate immunity, which might accelerate cellular senescence (Picca et al., 2019; Picca et al., 2022; Soto-Herederó et al., 2017). In older adults with physical frailty and sarcopenia, elevated EV levels containing mitochondrial components have provided additional evidence (Picca et al., 2020a). Studies also indicate EVs are more abundant in the serum samples of PD patients than in the age-matched control group without PD, indicating a mechanism for cells to dispose of damaged mitochondria (Singh et al., 2019). Besides, mitochondria couldn't secrete MDVs sufficiently in older PD patients, which may explain dysfunctional mitochondria lose their ability to degrade the misfolded proteins through MDVs in PD (Picca et al., 2020c). Hence, MDVs are like a double-edged sword, either delaying or accelerating aging. Understanding the role of MDVs in mitochondrial homeostasis may help discover new tools and strategies for preventing or treating age-related diseases.

In summary, MQC is a double-edged sword. They promote mitochondrial healthy by degrading harmful by-products and isolating damaged mitochondria. However, if MQC persists beyond a threshold, it can bring about negative consequences. Mitochondrial biogenesis can be hindered by proteasome competition for non-imported cytosolic precursor proteins (Kramer et al., 2021). Accumulation of misfolded or damaged proteins due to the over-activation of UPR^{mt} has been linked to aberrant cellular function and aging (Suarez-Rivero et al., 2022b). When both fission and mitophagy are overactive, cellular senescence may be accelerated (Yamamuro et al., 2020). Overloaded MDVs might release toxic by-products into the extracellular space (Popov, 2022). Therefore, selective, efficient, and gentle activation of MQC process is crucial for maintaining proper mitochondrial function. It implies that therapeutic interventions at various stages of MQC should be modulated appropriately. Additionally, the molecular and metabolism of mitochondria should be monitored simultaneously.

5. Therapeutic interventions on aging through targeting mitochondria

5.1. Targeting mitochondrial biogenesis

Strategies aimed at mitochondrial biogenesis generally include manipulating cellular biochemistry and energy homeostasis. Regular physical activity is known to increase mitochondrial biogenesis in various tissues. Exercise increases the demand for ATP, which signals the cell to produce more mitochondria to meet this increased energy demand (Huertas et al., 2019). High-intensity interval training can help prevent various metabolic diseases by increasing glucose utilization and can lead to an increase in cellular energy sensors, such as PGC-1 α , AMPK, SIRT1 and ROS in skeletal muscle (Torma et al., 2019). Studies have shown that exercise rescues age-related mitochondrial network fragmentation and restores impaired submaximal ADP-stimulated respiration (Halling et al., 2017). This occurs because exercise increases PGC-1 α expression and activity, which leads to an upregulation of mitochondrial biogenesis and function (de Oliveira Bristot et al., 2019; Halling et al., 2019).

AMPK is another crucial regulator of cellular energy homeostasis and is an upstream modulator of PGC-1 α . Due to its role as a central regulator of lipid and glucose metabolism, AMPK is a potential therapeutic target for treating age-related disorders, such as type-II diabetes mellitus, obesity, neurodegenerative diseases, and cancer (Lavu et al., 2008). Dietary restriction (DR) is described as a decrease in food intake that does not result in malnutrition. It has been found to delay the development of age-related diseases and prolong the lifespan of numerous creatures (Pearson et al., 2008). DR refers to intermittent fasting, a

fraction of the entire diet consumed by ad libitum-fed animals, or a limitation in a specific macro/micronutrient (Hou et al., 2016). Intermittent fasting has been proven to inhibit mTOR activity, decrease expression of Sirt3, and increase cellular NAD^+ /NADH which could activate AMPK/PGC-1 α (Brocchi et al., 2022; Curtis et al., 2022). Besides, a calorie restriction (CR) diet has also been demonstrated to improve mitochondrial biogenesis by activating AMPK (Niemann et al., 2022). It should be emphasized that DR levels beyond 60% may harm the organism's longevity and general health (Speakman and Mitchell, 2011).

Dietary supplements and potential drugs, known as calorie restriction mimetics or energy restriction mimetics, are proposed as a hypothetical class that would imitate the significant anti-aging benefits of CR. Resveratrol, a CR mimetic, increases muscle performance in old individuals by increasing mitochondria mass, and elevating Nrf-1 and TFAM (Rodríguez-Bies et al., 2016; Uddin et al., 2021). Metformin, the first-line anti-hyperglycemia for type 2 diabetes mellitus, is recently discovered as a blocker of complex I of the mitochondrial electron transport chain to decrease ATP levels and boost the AMP/ATP ratio, which in turn increases AMPK activation and decrease ROS production (Mohammed et al., 2021; Reid et al., 2020). However, Lilia et al. found metformin aggravates aging-related mitochondrial failure, resulting in lethal ATP depletion in aged worms and late-passage human cells (Espada et al., 2020). Thus, metformin therapy in later life may reduce cell survival and shorten longevity (Kodali et al., 2021). Rhizoma Cop-tidis, as an anti-aging natural product, has pharmacologic potential in combating aging-related diseases by activating AMPK cellular kinase (Xu et al., 2017). Rapamycin, an mTOR inhibitor, improves mitochondrial biogenesis and extends mice's lifespan (Liu et al., 2022). Thus, AMPK and mTOR might be critical mediators behind mitochondrial effects on longevity and, therefore, might provide a promising therapeutic target in treating aging-related diseases (Amorim et al., 2022).

5.2. Targeting mitochondrial dynamics

Dynamics in mitochondrial shape and networks impact on mitochondrial function and quality. Studies have proved that co-inhibition of fusion and fission by intermittent fasting could reduce the lifespan of yeast (Weir et al., 2017). In comparison, 20% of female and 12% of male flies lived longer than their typical lifespan, if induced with DRP1-mediated fission in the midlife of *Drosophila melanogaster* (Rana et al., 2017).

A recent study also implies that exercise improves muscle function by regulating mitochondrial dynamics via the AMPK pathway (Campos et al., 2023). Impairing mitochondrial fission or fusion negates the beneficial effects of constitutive AMPK activation on physical fitness preservation in older people (Campos et al., 2023). Resveratrol is generally used as a dietary supplement. It suppresses mitochondrial fusion and promotes fission through the PINK1/Parkin pathway (Ren et al., 2017). A study shows, however, resveratrol inhibits both fusion and fission to modulate mitochondrial quality in the diabetic muscle of mice (Wang et al., 2018a). Therefore, strategies for modulating the balance of mitochondrial fission and fusion appear critical for anti-aging outcomes.

5.3. Targeting UPR^{mt}

Previous studies have discovered that activation of UPR^{mt} contributes to longevity (Zhu et al., 2021). For instance, ROS elimination by activating the effector of UPR^{mt} -ClpP delays liver cell senescence (Sen et al., 2019). Study on Honokiol (HKL, $C_{18}H_{18}O_2$), a pharmacologically active natural compound isolated from the bark of *Magnolia officinalis*, showed that HKL activates sirtuin 3 (SIRT3)- UPR^{mt} signaling and improves cognitive impairment in a mouse model of AD (Hou et al., 2022). UPR^{mt} is therefore a possible therapeutic target. It is also reported that protein disulfide isomerase 6 (PDI-6) is expressed in the endoplasmic

reticulum and interacts with EGL-20 through disulfide bonds. Over-expression of PDI-6 through UPR^{mt} activation is sufficient to keep Wnt/EGL-20 protein levels stable during aging in *C. elegans* (Li et al., 2022). Still, the long-term effects of this activation remain uncertain because prolonged UPR^{mt} activation can result in mitochondrial dysfunction, and the transcription of numerous unknown functional genes may induce cancer (Lin et al., 2016; Suarez-Rivero et al., 2022a).

5.4. Targeting mitophagy

Pharmacological therapies targeting mitophagy have generated considerable interest and may have significant translational potential. By demonstrating a protective function for mitophagy in neurons and microglia, a study raises the possibility that mitophagy induction is advantageous for other glial cells and non-neuronal cells (Fang et al., 2019b). Several strategies are applied to modify mitophagy signaling pathways or directly target mitophagy proteins. Some evidence indicates that increased mitophagy through activation of the PINK1/Parkin pathway is a promising therapeutic target for PD (Pickrell and Youle, 2015). Metformin is a natural SIRT1-activating compound with an anti-aging property by activating PINK1/Parkin-mediated mitophagy (Wang et al., 2019b). Rapamycin promotes mitophagy by inhibiting mTOR and elevating PINK1, Parkin, and the pro-autophagic protein BECN1 (Mahalakshmi et al., 2022). Besides, Atgs play a vital role in autophagosome formation. Atg32 is one of the mitophagy-specific receptors anchored on the OMM. It acts with Atg11 and Atg8 at the phagophore assembly site for lysosome degradation (Okamoto et al., 2009). In addition, Atg2-Atg18/Atg9 complex could induce mitophagy to keep mitochondrial integrity in *Drosophila* (Chumpen Ramirez et al., 2022). Several natural plant extractions and postbiotic metabolites have been shown to promote mitophagy in recent years. *Gentiana iridescens*, a biennial herbaceous species, extends the lifespan of yeast by activating Atg32 gene to promote mitophagy (Liu et al., 2020). *Lepidium meyenii*, a relative of the radish, improves cognitive function in middle-aged mice by upregulating autophagy-related proteins, such as LC3, Atg7, and Beclin1 (Guo et al., 2016). Urolithin A is a polyphenolic molecule found in pomegranate, cherries, and walnuts as a postbiotic metabolite. In pre-clinical models of aging and age-related diseases, urolithin A has been demonstrated to stimulate mitophagy and improve mitochondrial function. This finding indicates Urolithin A improves muscle strength, exercise performance, and indicators of mitochondrial health by activating mitophagy in a randomized clinical trial in middle-aged people (Singh et al., 2022a). Oleanolic acid is a pentacyclic triterpenoid found in nature, that has been shown to have antioxidant and cyclooxygenase (P450) modulating effects. Treatment with oleanolic acid attenuates aging-related alterations in mitophagy via regulating LC3B, p62, TOM20, and FUNDC1, but not BNIP3 or Parkin (Gong et al., 2022). Kaempferol and Rhapontigenin, two mitophagy inducers, enhance memory in nematode and rodent models of AD by increasing the survival and functioning of glutamatergic and cholinergic neurons and decreasing A β and tau pathologies (Xie et al., 2022). Besides, exercise is proven to boost mitochondrial fission and mitophagy through the PGC1 α /FNDCC5/irisin pathway (He et al., 2020). Through FUNDC1-dependent mitophagy, exercise shields elderly mice from coronary endothelial senescence (Ma et al., 2023). It is also found that both short-term and long-term exercise trainings cause changes in the levels of LC3-II and the ubiquitin-binding protein p62 (Joseph et al., 2016). However, the pathways responsible for these exercise-induced mitochondrial adaptations remain controversial, as other studies have reported a decreased adaptability of old muscles to exercise training compared to young (Grevendonk et al., 2021; Triolo et al., 2022). Taken together, maintaining moderate regulation of mitophagy is crucial for the regulation of aging processes.

Table 1
Interventions on aging through targeting mitochondria.

Target	Interventions	Mechanism of action	References
Mitochondrial biogenesis	Exercise	Stimulation of PGC-1 α and AMPK	(Torma et al., 2019; Halling et al., 2017; de Oliveira Bristot et al., 2019; Halling et al., 2019)
	Intermittent fasting	Inhibition mTOR activity Decreased expression of Sirt3 Increased cellular NAD ⁺ /NADH ratio	(Brocchi et al., 2022) (Curtis et al., 2022)
	Calorie restriction	Activation of AMPK	(Niemann et al., 2022; Speakman and Mitchell, 2011)
	Resveratrol	Increases mitochondrial mass and elevating Nrf1 and TFAM	(Rodriguez-Bies et al., 2016; Uddin et al., 2021)
	Metformin	Activation of AMPK	(Mohammed et al., 2021; Reid et al., 2020)
	Rhizoma Coptidis Rapamycin	Activation of AMPK Inhibition of mTOR	(Xu et al., 2017) (Liu et al., 2022)
Mitochondrial dynamics	Intermittent fasting	Co-inhibition of fusion and fission Regulation of mitochondrial fission, fusion	(Weir et al., 2017)
	Exercise	AMPK activation Promotion of fission and suppression of fusion via PINK1/Parkin pathway	(Campos et al., 2023)
	Resveratrol	Inhibition of both fusion and fission	(Ren et al., 2017)
	Resveratrol	Inhibition of both fusion and fission	(Wang et al., 2018a)
UPR ^{mt}	Honokiol	Activation of SIRT3-UPR ^{mt}	(Hou et al., 2022)
	PDI-6	Activation of Wnt / EGL-20	(Li et al., 2022)
Mitophagy	Metformin	Enhancing the SIRT1 /PINK1 /Parkin pathway Inhibiting mTOR	(Wang et al., 2019b)
	Rapamycin	Elevation of PINK1, Parkin and BECN1	(Mahalakshmi et al., 2022)
	Atgs	Activation of Atg32/Atg11/Atg9	(Chumpen Ramirez et al., 2022)
	<i>Gentiana iridescens</i> <i>Lepidium meyenii</i>	Activation of Atg32	(Liu et al., 2020)
	Urolithin A	Upregulating LC3, Atg7, and Beclin1 Activation of PINK1/Parkin pathway	(Guo et al., 2016) (Singh et al., 2022a)
	Oleanolic Acid	Modulating of FUNDC1, LC3B, p62, TOM20	(Gong et al., 2022)
	Kaempferol Rhapontigenin	Activation of PINK1/Parkin pathway Modulating FUNDC1, LC3-II, p62	(Xie et al., 2022) (Gong et al., 2022; Ma et al., 2023)
	Exercise	Activation of PGC1 α /FNDCC5/irisin pathway Modulation of LC3-II and p62	(He et al., 2020) (Joseph et al., 2016)

(continued on next page)

Table 1 (continued)

Target	Interventions	Mechanism of action	References
MDVs	Cannabidio	Activation of PINK1/Parkin pathway	(Ramirez et al., 2022)
	Mitochondria-rich EVs	Restoration of mitochondrial biogenesis via PGC-1 α	(Urabe et al., 2020)

5.5. Targeting MDV transportation

The role of MDVs in aging is still debatable. On one hand, intracellular MDV formation as earlier mitochondrial quality control is cyto-protective. Evidence indicates MDVs protect cardiomyocytes against apoptosis and ameliorate hypoxia-induced damages (Li et al., 2020). Ramirez et al. discovered cannabidiol, a drug for neurological disorders, could induce the production of MDVs by activating the PINK/Parkin pathway dose-dependently and repair mitochondrial damage through opening permeability transition pores (Ramirez et al., 2022). On the other hand, MDVs could be released into the extracellular space (Picca et al., 2020b). Although whole mitochondria in extracellular vesicles are proven to improve cardiac function in post-myocardial infarction animal models (Ikeda et al., 2021), the destination and influence of the MDV-rich EVs (MDV-EVs) are still obscure. To date, EV-targeting therapeutic strategies include inhibiting EV secretion and absorption, as well as eliminating EV circulation (Urabe et al., 2020). Given the critical role

of MDVs in regulating mitochondrial quality control, there is growing interest in targeting MDV-EVs as a potential therapeutic strategy for anti-aging therapy. By manipulating MDV-EVs' content, it may be possible to modulate cellular function and mitigate the effects of aging-related changes.

Due to the multifaceted characteristics of neurodegenerative diseases, more comprehensive therapeutic interventions may be required. To control the pandemic of neurological disorders in the elderly, it is necessary to combine non-pharmacological methods and lifestyle modification with multi-targeted pharmaceutical therapies (Hou et al., 2019).

6. Conclusions and prospects

A growing body of evidence implicates MQC provides new avenues for therapeutic intervention in aging and age-related disorders (Guo et al., 2022). Table 1 summarises the various anti-aging approaches that target mitochondria quality modulation. Although mitochondrial dysfunction plays a role in the pathophysiology of age-related disorders, imbalanced MQC has only recently been identified as a critical contributor to disease pathogenesis (Sharma et al., 2021). Therefore, the role of MQC must be elucidated to comprehend the mechanism of aging from the perspective of various fields of mitochondrial maintenance in response to stress. For instance, mitochondrial proteases may serve as the frontline of defence against misfolded proteins (Smakowska et al., 2014), while UPR^{mt} aids in refolding misfolded proteins by communicating with the nucleus, in which the target genes also modulate mitochondrial proteases, dynamics, and mitophagy (Ji et al., 2020). Although MDVs and mitophagy share similar molecular modulators

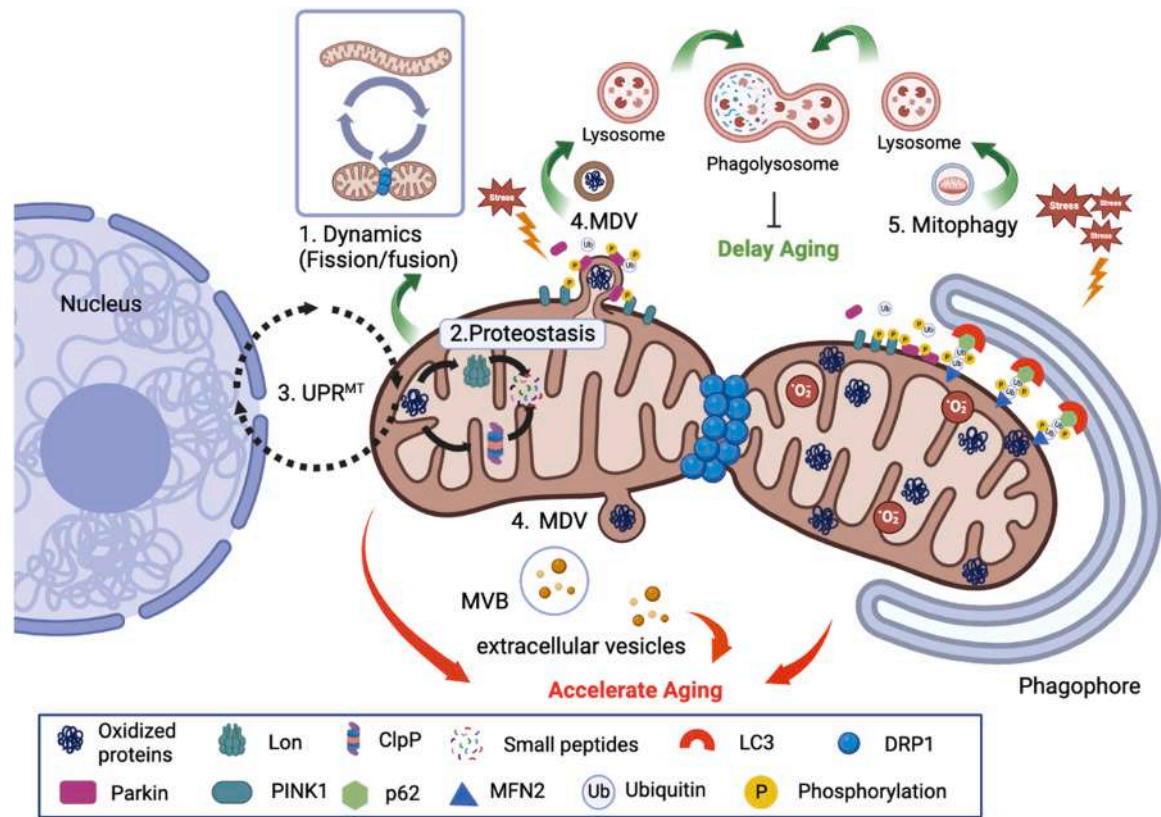


Fig. 7. Mitochondrial quality control and aging. Mitochondria are highly dynamic organelles that endure coordinated cycles of fission and fusion. Mitochondrial proteases and UPR^{mt} maintain mitochondrial proteostasis through refolding the misfolded proteins or proteolysis into small peptides. Oxidative stress activates PINK1/Parkin-dependent pathway to induce MDV formation and mitophagy. On the other hand, single/double membrane MDVs could deliver the oxidized by-products to lysosomes in physical condition or under mild oxidative stress. On the other hand, severe or prolonged oxidative stress may induce mitochondrial depolarization and mitophagy. However, overactivation or inhibition of any form of MQC may accumulate oxidized proteins, accelerate aberrant energy metabolism, and induce mitochondrial dysfunction-induced senescence.

(PINK1 and Parkin), they are found in different conditions. Damaged proteins can be disposed of via MDVs, or the recycling of partial or entire organelle via mitophagy (Roberts et al., 2016). Formation of MDVs may compensate for the loss of mitophagy to maintain mitochondrial homeostasis (Towers et al., 2021). MDVs could also be released as exosomes and transported to the adjacent cells to trigger innate immunity, which might accelerate cellular senescence (Picca et al., 2019; Picca et al., 2022; Soto-Herederó et al., 2017). Thus, the relationship between MDVs and mitophagy in aging is still obscure. Either excessive activation or insufficient of MQC might hasten the aging process (Fig. 7). Furthermore, the function of mitochondria may not decline linearly throughout the aging process. The activity of mitochondria in middle-aged individuals demonstrates a compensatory increase after the disposal of damaged mitochondria, whereas mitochondrial dysfunctions develop in the aged population (Baker and Peleg, 2017). Therefore, maintaining functional mitochondria is essential for long-term health.

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Author contributions

Y.G. drafted the original manuscript. T.G., K.S., Q.Y., X.J., D.N., and M.L. reviewed and edited the manuscript. G.Z. and J.K. revised and approved the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

We declare no conflict of interest.

Data availability

Data will be made available on request.

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Carbon monoxide preconditioning is mediated via activation of mitochondrial-derived vesicles

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ABSTRACT

Preconditioning with inhalative carbon monoxide (CO) at low concentrations provides protection against hypoxic and ischemic insults in the brain and heart. The present study aims to test a hypothesis that activation of mitochondrial-derived vesicles (MDVs) is a mechanism underlying the protective effect of CO preconditioning. Here we show that CO preconditioning induced mild oxidative stress and activated massive production of MDVs. Short exposure to a low concentration of carbon monoxide-releasing molecule 2 (CORM-2), a donor of carbon monoxide, prevented oligodendrocyte precursor cells (OPCs) from subsequent death induced by high doses of CO, and protected Chinese hamster ovary (CHO) cells against oxygen-glucose deprivation (OGD)-induced cell death. Furthermore, inhibition of lysosomal activity prevented degradation of MDVs, abolished MDV-mediated mitochondrial quality control, and diminished the protective effect of CO preconditioning. Altogether, our data provide direct evidence suggesting that MDV-mediated mitochondrial quality control may have a novel role in CO preconditioning.

1. Introduction

Carbon monoxide (CO) is well-known for its toxic properties due to its high affinity to hemoglobin (Tian et al., 2020). As an gaseous signaling molecule, CO is implicated with important biological functions. CO at low or near-physiological doses has been shown to be protective by that limits neuronal apoptosis and leads to protection against permanent focal cerebral ischemia (Wang X.Q. et al., 2011; Wang B. et al., 2011; Queiroga et al., 2012). Preconditioning with inhalative CO at low concentrations induces a late preconditioning-mimetic cardioprotective and antiapoptotic milieu in

the myocardium (Queiroga et al., 2012; Stein et al., 2012). The mechanisms by which CO preconditioning protects cells are not fully understood although generation of reactive oxygen species, stabilization of hypoxia-inducible factor 1 α and inhibition of apoptotic pathways are suggested (Chin et al., 2007; Wang et al., 2018).

Mitochondria are one of the known targets for CO. Mitochondria are highly dynamic organelles that are critical for energy metabolism of the cell. Mitochondrial dysfunction has been implicated in ageing and a variety of ageing-related diseases. The quality of mitochondria is maintained through a series of well-coordinated processes (Picca et al., 2018). Mitochondrial proteases in the mitochondrial matrix and

Abbreviations: AA, Antimycin A; AIF, Apoptosis-inducing factor; CCCP, Carbonyl cyanide m-chlorophenylhydrazone; CHO, Chinese hamster ovary cells; CO, Carbon monoxide; CORM-2, Carbon monoxide-releasing molecule-2; DNS, Delayed neurologic sequelae; EM, Electron microscopy; iCORM-2, Inactive CORM-2; MDVs, Mitochondrial-derived vesicles; MQC, Mitochondrial quality control; Na₂S₂O₄, Sodium dithionite; NH₄Cl, Ammonium chloride; OPCs, Oligodendrocytes; OGD, Oxygen-glucose deprivation; ROS, Reactive oxygen species; SIM, Super-resolution structured illumination microscopy; TOM20, Translocase of the outer membrane 20; PDH, Pyruvate dehydrogenase; Stx17, Syntaxin 17; PDGF-AA, Platelet-derived growth factor-AA; PDGFR α , Platelet-derived growth factor receptor α ; b-FGF, Basic-fibroblast growth factor; PI, propidium iodide.

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intermembrane space are the first line of defense to maintain proteostasis against unfolded and oxidized soluble proteins (Szczepanowska and Trifunovic, 2021). Oxidized outer membrane proteins are removed via the proteasome pathway following ubiquitination (Sulkshane et al., 2021). Upon dysfunction, a whole mitochondrion can be targeted to the autophagosome and removed through mitophagy (Chakravorty et al., 2019). In certain conditions, mitochondria remove their damaged patches through the generation of mitochondrial-derived vesicles (MDVs), which transit to the late endosomes and fuse with lysosomes for degradation (Neuspiel et al., 2008; Ryan et al., 2020; Soubannier et al., 2012). The formation of MDVs is an earlier response to oxidative stress and a major approach to removing oxidized proteins and lipids from mitochondria (Cadete et al., 2016; McLelland et al., 2014). The present study aims to determine the extent to which activation of MDVs is involved in the protective effect of CO preconditioning.

2. Materials and methods

2.1. Cell culture and transfection

Chinese hamster ovary (CHO) cells (ATCC, US, cat. CCL-61 m RRID: CVCL_0241) were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Fisher, cat. 11960069) supplemented with sodium pyruvate (ThermoFisher, cat. 11360070), penicillin/streptomycin (Fisher, cat. 15640055), and 10% fetal bovine serum (Gibco, cat. LS26140079) with 5% CO₂ at 37 °C. Cells were maintained in the culture medium for at least 1 week before the experiment and the maximum number of passages for CHO cell line was 30. The plasmids (YFP-Stx17) were kindly provided by Dr. Edward A. Fon's Lab (McGill University, Montreal). CHO cells (5×10^4) were plated into a live-cell chamber (Nunc, lab-Tek, cat. 155383) for 24 h and then transfected with the YFP-Stx17 with lipofectamine 2000 (Fisher, cat. 11668027), 24 h before being used for further experiment.

Primary oligodendrocyte precursor cells were prepared from newborn (P1) Sprague Dawley rats with both sexes as described previously (Zhang et al., 2020). All animal experiments were performed in accordance with the Guide of Care and Use of Experimental Animals of the Canadian Council on Animal Care. Timed pregnant female rats were obtained from Central Animal Care Services, University of Manitoba (Protocol #: B2020–029). Briefly, rat pups were sacrificed by decapitation. Cerebral cortices were dissected and mechanically dissociated. The cells were then grown in DMEM supplemented with 20% fetal bovine serum (Thermo Fisher Scientific, Eugene, OR, USA). After 10 days in culture, flasks containing the cells were shaken at 220 rpm for 1 h to remove microglia. The cultures were then shaken at 220 rpm overnight to dislodge loosely attached oligodendrocyte precursor cells (OPCs). OPCs were further purified from astrocytes and microglia by being placed in uncoated tissue culture dishes for 1 h. The non-adherent cells were collected and cultured in a chemically defined medium containing 4 mM L-glutamine, 1 mM sodium pyruvate, 0.1% bovine serum albumin, 50 µg/mL Apo-transferrin, 5 µg/mL insulin, 30 nM sodium selenite, 10 nmol/L D-biotin, and 10 nM hydrocortisone. This chemically defined medium was supplemented with 10 ng/mL PDGF-AA (Peprotech, Cat. 100–13 A) and 10 ng/mL bFGF (PeproTech, Cat. 100–18B). The purity of OPCs in culture was assessed by immunolabeling of OPCs with an antibody to PDGFRα (1: 1000, Cell Signaling, cat. 3164S).

2.2. Preparation of CORM-2 and iCORM-2

Carbon monoxide-releasing molecules (CORMs) are chemical compounds that release carbon monoxide (CO) under controlled conditions. Tricarbonyldichlororuthenium (II) dimer (CO-releasing molecule-2, CORM-2, Sigma cat. 288144, Oakville, Ontario, Canada) was dissolved in 50% DMSO to make a 50 mM stock solution and stored at – 80 °C. The final concentration of DMSO in samples was less than 0.5%. Inactive

CORM-2 (iCORM-2) was prepared by leaving CORM-2 stock solution at 37 °C in a humidified 5% CO₂ atmosphere to liberate CO for 24 h before use.

2.3. Assessment of cytotoxicity

Cells (1×10^4 /mL) were seeded in a 96-well plate at a final volume of 100 µL/well. After 24 h, the cells were exposed to CORM-2 at concentrations ranging from 5 to 100 µM for 24 h. Then, 10 µL of WST-1 (VWR, Cat. 10180–420) was added to each well. After incubation for 4 h, the absorbance was measured on a Bio-Rad microplate reader at 562 nm.

2.4. Assessment of apoptosis

A total of 2.5×10^4 cells were seeded on Poly-D-Lysine (PDL) coated coverslips in a 24-well plate. After accordant treatment, the cells were washed with PBS and then incubated with propidium iodide (PI, 1:500, Sigma, Cat. P4170) and Hoechst 33258 (Sigma, cat. 94403) for 15 min at 37 °C. After brief washing in PBS, the cells were fixed with 4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature, and the coverslips were mounted with a drop of fluorescence mounting medium (Dako, Carpinteria, CA 93013, USA). The cells were examined on a Zeiss Axio Imager Z2 microscope.

2.5. Mitochondrial morphology analysis

Cells were incubated with MitoTracker Red (Invitrogen, cat. M7512) at 37 °C for 30 min, washed in pre-warmed PBS, and fixed with 4% formaldehyde for 15 min. Morphology of mitochondria was evaluated by two indexes: average circularity and interconnectivity (the ratio of area to perimeter) using the Image J software (1.53c) as described previously (Dagda et al., 2009). An increase in average circularity and a decrease in interconnectivity indicate poor mitochondrial quality.

2.6. Measurement of mitochondrial membrane potential

Cells were stained with the JC-1 dye (Thermo Fisher, Cat. T3168) for 10 min. After washing in PBS twice, the cells were examined for fluorescence intensity of JC-1. Cells treated with carbonyl cyanide m-chlorophenylhydrazine (CCCP, Sigma, cat. C2759) at 50 µmol for 10 min were used as a positive control. The images were analyzed with the Image J software.

2.7. ROS production in cells and mitochondria

The H₂DCFDA (Thermo Fisher, cat. D399) kit was employed to test the total cellular ROS production. The fluorescence signals were tested according to the manufacturer's protocol. The Mito-SOX (Thermo Fisher, cat. M36008) kit was used to test superoxide production. Cells were incubated with Mito-SOX working solution according to the manufacturer's protocol and imaged by Axio observer D1 (Zeiss) microscope.

2.8. Immunofluorescence

Cells cultured on coverslips in 24-well plates were fixed in 4% formaldehyde for 15 min. The cells were then washed with PBS, permeabilized in 0.25% Triton-X 100/ PBS (PBST) for 10 min and blocked with 1% bovine serum albumin in PBST for 30 min. Cells were then incubated with primary antibodies at 4 °C overnight, washed 3 times with PBS, then incubated with appropriate secondary antibodies at room temperature for 1 h. Both primary and secondary antibodies were diluted in 1% bovine serum albumin in PBST. The nuclei were counterstained with Hoechst 33258. Finally, the cells on coverslips were washed 3 times before being mounted on glass slides. Images were acquired on a Zeiss AXIO Imager Z2 Motorized Fluorescence Microscope or

a ZEISS Elyra PS.1 super-resolution structured illumination microscopy (SIM). MDVs were identified after immunolabeling with antibodies to TOM20 (Abcam, cat. ab186735) and PDH E2/E3 (Abcam, cat. ab110333). The size of the MDV puncta was around 300 nm under immunofluorescence microscope due to the lateral resolution, however, the actual size was 70–150 nm under EM (Cadete et al., 2016). MDVs were counted manually in 50 cells per experiment. Quantification of AIF nuclear translocation rate was achieved with image J software. A total of 50 cells in each group from 3 independent experiments were analyzed. For live cell imaging, the YFP-Stx17 transfected cells were stained with MitoTracker Red (Invitrogen, cat. M7512) and Mito-SOX (Thermo Fisher, cat. M36008) for 30 min, and Hoechst33258 for 10 mins. After washing with PBS twice, live-cell images were acquired on a Zeiss LSM700 Spectral Confocal Microscope in a 37 °C-chamber containing 5% CO₂.

2.9. Electron microscopy (EM)

Cells were fixed in 4% glutaraldehyde and washed with PBS. The cells were then post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences, cat. 19152) in 0.1 M Sorensen's buffer for 1 h and washed. Following dehydration, infiltration, embedding and polymerization, samples were cut and collected onto copper grids, and stained in uranyl acetate (Electron Microscopy Sciences, cat. 22409) and lead citrate (Electron Microscopy Sciences, cat. 22410). Cell samples were observed on a Philips 201 transmission electron microscope.

2.10. Statistical analysis

Data were presented as means $\pm$ SD. Statistics were performed using GraphPad Prism 9.0. To determine the distribution of the data, a Shapiro-Wilk normality test was utilized. In the case of a non-normal distribution, a Kruskal-Wallis test was used to test statistical significance. For data with normal distribution, a one-way ANOVA with Dunnett's test of multiple comparisons was performed to determine differences between multiple treatments. Data were considered statistically significant when $p < 0.05$.

3. Results

3.1. CO preconditioning provides protection against CO poisoning in oligodendrocyte precursor cells (OPCs)

Previously we showed evidence for direct toxicity of CO to neurons and glial cells at high doses. Oligodendrocytes are particularly susceptible to CO (Tian et al., 2020). In the present study, we used primary rat OPCs with a purity of $> 90\%$ as determined by immunofluorescence staining for PDGFR α (Fig. 1a). Carbon monoxide-releasing molecule 2 (CORM-2) is a donor for carbon monoxide. It becomes toxic to OPCs at concentrations higher than 100 μM (Tian et al., 2020). To determine if a low dose of CO provides preconditioning effects, OPCs were exposed to CORM-2 (Sigma, 288144) at 2.5 μM for 2 h and then challenged with CORM-2 at 500 μM for 6 h. OPCs had fewer branches and were less connected in the 500 μM CORM-2 group than other groups of cells (Fig. 1b). About 20% of the cells treated with CORM-2 at 500 μM for 6 h

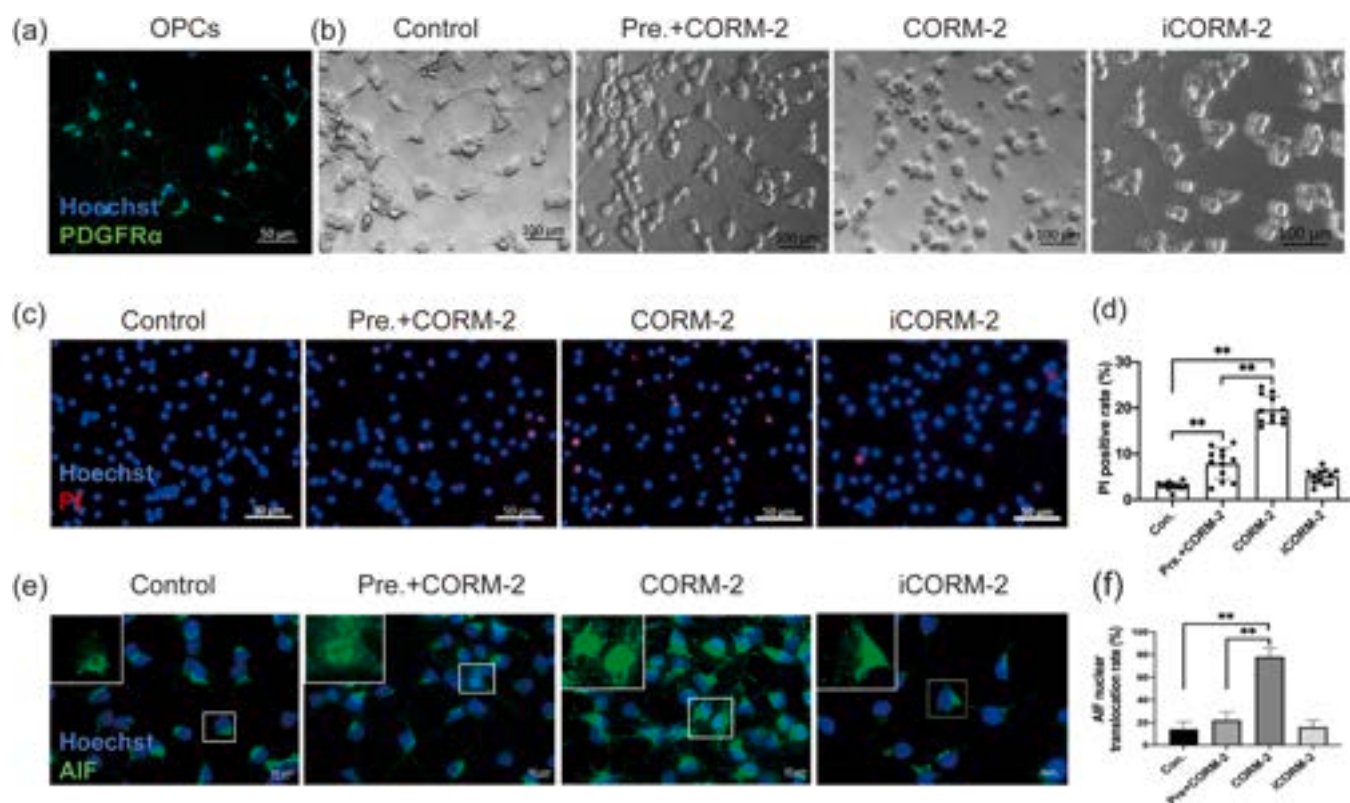


Fig. 1. CO preconditioning inhibits apoptosis of OPCs. (a) Oligodendrocytes were stained with PDGFR α (Green) and Hoechst (Blue) to evaluate the enrichment of OPCs. Scale bar = 50 μm . (b) Cell morphology was observed under light microscope. OPCs were pretreated with 2.5 μM CORM-2 for 2 h before 500 μM CORM-2 for 6 h (Pre. + CORM-2) and were only treated with 500 μM CORM-2 for 6 h (CORM-2 group) or 500 μM iCORM-2 for 6 h (iCORM-2 group). OPCs without any treatment were taken as control. Scale bar = 100 μm . (c) The apoptotic OPCs were shown colocalization of PI (Red) with Hoechst (Blue). Scale bar = 50 μm . (d) Quantification of apoptotic OPCs by calculating the percentage of PI-positive cells (DF=3, F=112.7, $^{**}P < 0.01$), n = 3 independent experiments. (e) OPCs were stained with anti-AIF (Green) and Hoechst (Blue) in control, Pretreated+CORM-2, CORM-2, and iCORM-2 groups. The enlarged area was shown as the green signal channel of AIF. Scale bar = 10 μm . (f) Percentage of cells with nuclear translocation of AIF was calculated from 50 cells per group (DF=3, F=19.28, $^{**}P < 0.01$) n = 3 independent experiments. Statistical analysis was conducted using one-way ANOVA.

showed remarkable signs of cell death as detected by propidium iodide (PI, Sigma, P4170) staining (Fig. 1c). Preconditioning with CO at 2.5 μ M for 2 h significantly reduced the PI-positive OPCs induced by CORM-2 at 500 μ M for 6 h (Fig. 1d). To determine if the dying cells were apoptotic, we tested nuclear translocation of apoptosis-inducing factor (AIF) by immunofluorescence staining with a primary antibody of AIF (Santa Cruz sc-13116). The results showed that CORM-2 at 500 μ M for 6 h induced nuclear translocation of AIF in nearly 80% of the cells. Preconditioning with CO robustly inhibited AIF translocation (Fig. 1e and f).

3.2. CO preconditioning induces massive production of MDVs in OPCs without interfering with mitochondrial morphology and membrane potential

To investigate possible mechanisms by which CO preconditioning protects OPCs, we examined generation of MDVs in OPCs. MDVs were identified after immunolabeling with antibodies to TOM20 (Abcam, ab186735) and PDH E2/E3 (Abcam, ab110333). TOM20 is a mitochondrial outer membrane protein, and PDH is in the mitochondrial matrix. MDVs were identified by cargo selectivity (either TOM20-positive or PDH-positive, but not both) under immunofluorescence microscope as described previously (Soubannier et al., 2012). The two types of selective cargos were used to distinguish MDVs from mitochondrial main structure (which are both positive). MDVs were shown as puncta adjacent to mitochondria in OPCs (Fig. 2a). We found that treatment with CORM-2 at 2.5 μ M for 2 h in OPCs induced significantly more MDVs than control groups without affecting mitochondrial circularity (Fig. 2, b and c). At 5 h of exposure to CORM-2, the number of MDVs reduced to the level of the control while the mitochondrial circularity increased significantly, and mitochondria became depolarized as revealed by a significant decrease of the red/green ratio of the JC-1 fluorescence intensity (Fig. 2, d and e).

3.3. Generation of MDVs is an early mitochondrial response to oxidative stress

To further verify if mild oxidative stress triggers formation of MDVs, we treated CHO cells with antimycin A (Sigma, A8674), a mitochondrial complex III inhibitor. As shown in Fig. 3a, antimycin A at 10 μ M induced massive formation of MDVs as revealed by immunofluorescence labeling of mitochondria with TOM20 and PDH. MDVs were also checked by electron microscopy (Fig. 3b). MDVs increased in number robustly and peaked by 2 h after antimycin A was applied, indicating that the formation of MDVs was an early response to mild oxidative stress (Fig. 3c). The number of MDVs reduced back to basal level in 5 h. Syntaxin-17 (Stx17), an essential transporter of MDVs, was found to localize in PDH-positive but not in TOM20-positive MDVs (Fig. 3d). Live cell imaging with MitoTracker Red further demonstrated that MDVs in cells treated with antimycin A were robustly induced, and each MDV lasted less than 120 s (Fig. 3e).

3.4. MDV formation causes no detectable morphological changes or depolarization of mitochondria in CHO cells

We next analyzed effects of MDV formation on mitochondrial structure and function. With MitoTracker Red (Fig. 4a) labelling, the circularity and interconnectivity of mitochondria were calculated with the Image J software. The results showed that there were no significant changes in circularity and interconnectivity of mitochondria in cells treated with antimycin A (10 μ M) for 2 h. Cells treated for a longer time (10 μ M, 5 h) increased mitochondrial circularity and reduced interconnectivity (Fig. 4b and c).

To further find out whether MDVs were required for maintaining mitochondrial health, the JC-1 dye was applied to monitor mitochondrial membrane potential. JC-1 acts as green-fluorescent J-monomers

but accumulates in mitochondria as red fluorescent J-aggregates in a potential-dependent manner. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The data shows there were no significant changes in the red/green ratio within 3 h when CHO cells were treated with antimycin A, however, the ratio dropped dramatically when cells were treated longer than 4 h (Fig. 4d and e). The results indicate that mild oxidative stress in a short time does not affect the mitochondrial structure and function, while a stronger or prolonged oxidative stress induced mitochondrial morphologic changes and depolarization.

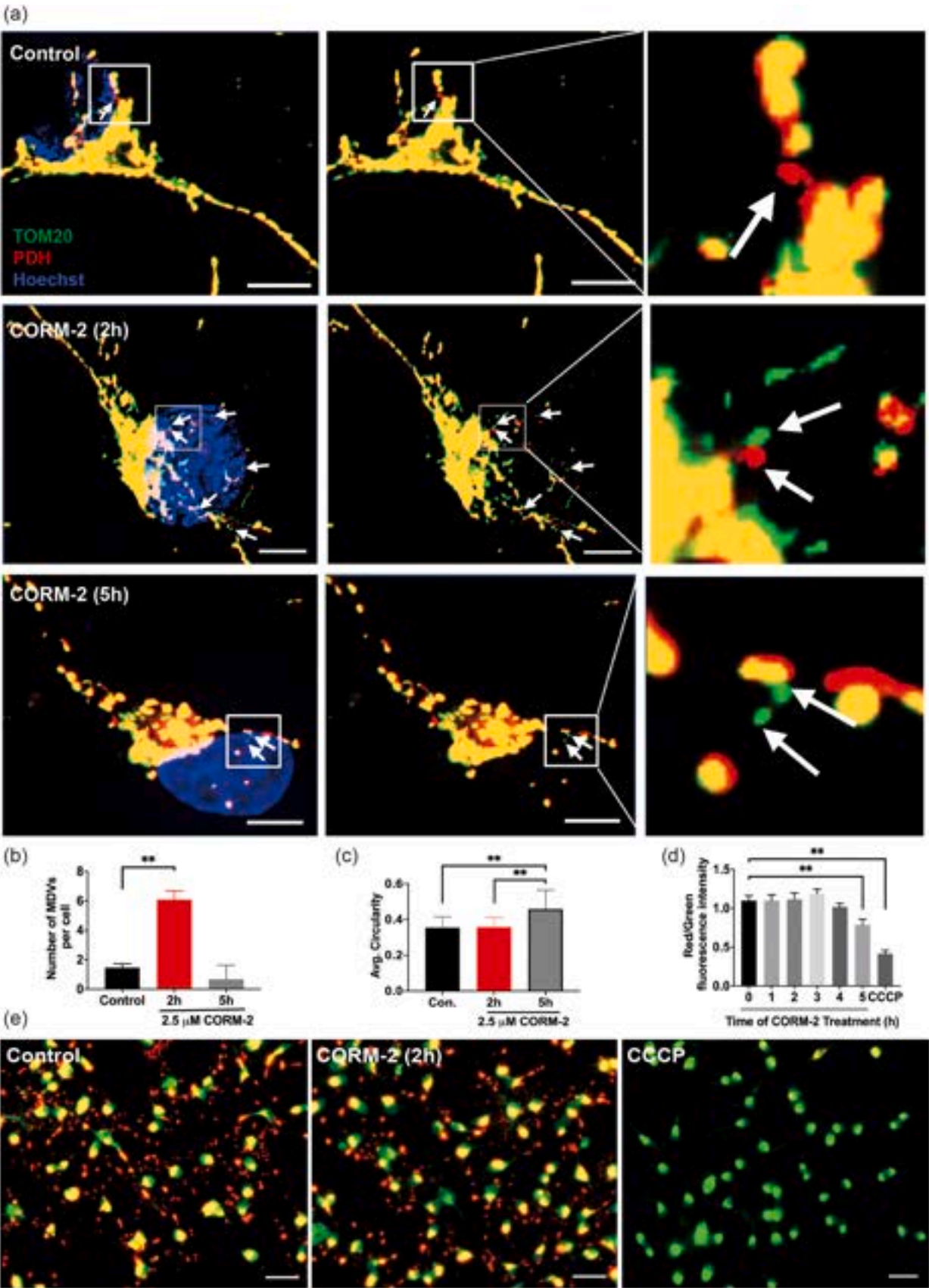
3.5. CO preconditioning protects cells against severe oxidative stress through generation of MDVs

Generation of reactive oxygen species is known to be necessary in CO preconditioning and neuroprotection (Vieira et al., 2008). To verify protective effects of CO preconditioning against cell death induced by oxygen-glucose deprivation (OGD), we treated CHO cells in culture with sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), an oxygen scavenger, in glucose-free EBSS (Fig. 5a). Since the IC_{50} of $\text{Na}_2\text{S}_2\text{O}_4$ was 11.38 mM, $\text{Na}_2\text{S}_2\text{O}_4$ at 10 mM for 3 h was used to challenge CHO cells. The results showed that preconditioning with 60 μ M of CORM-2 had the lowest death rate among all groups ($P < 0.01$) (Fig. 5b). Preconditioning with 60 μ M CORM-2 could stimulate ROS production in mitochondria shown in Mito-SOX staining but significantly decreased the total ROS production induced by OGD as detected with the H_2DCFDA (Thermo Fisher, D399) assay ($P < 0.01$) (Fig. 5, c and d). The results indicate that CO preconditioning alleviated the damage to mitochondria under severe oxidative stress. Immunolabeling with antibodies to PDH and TOM20 showed no significant changes in mitochondrial morphology but the presence of numerous single labeled puncta that were typical of MDVs in control, 60 μ M CORM-2 (2 h), and 60 μ M CORM-2 (2 h) plus 10 μ M ammonium chloride (NH_4Cl) (2 h) (Fig. 5e). Inhibition of lysosomal activity with 10 μ M NH_4Cl caused increased circularity of mitochondria and accumulation of MDVs (Fig. 5, f and g). PI positive cells increased in CORM-2 preconditioned together with NH_4Cl treatment group under OGD condition, suggesting that lysosomal inhibition counteracted CO-preconditioning's protective effects (Fig. 5, h and i).

4. Discussion

It is well established that CO preconditioning provides protection against hypoxic and ischemic insults in the brain and heart (Queiroga et al., 2012; Stein et al., 2012). The present study offers a mechanism for the protective effects of CO preconditioning. We found that CO at low concentrations induces massive production and release of MDVs. Since the release of MDVs is a primary pathway for mitochondrial quality control in response to mild oxidative stress, our results support that activation of MDV-mediated mitochondrial quality control underlies the protective effects of carbon monoxide preconditioning.

Accumulation of ROS leading to oxidative stress is known to be one of the main mechanisms for mitochondrial dysfunction (Eckert et al., 2003). It is interesting to note that MDVs could mitigate cellular total ROS levels. On the other hand, a certain level of ROS under mild oxidative stress is required for the protective effect of CO preconditioning (Vieira et al., 2008). The protective effect of CO preconditioning involves the activation of mitochondrial quality control (MQC) to replace defective mitochondria or eliminate mitochondrial debris, preserving the structure and function of the network of these organelles, and consequently ensuring homeostasis and survival of cells. MDVs are recently discovered as an upstream event of MQC and an earlier response to oxidative stress than mitophagy (Cadete et al., 2016; McLelland et al., 2014). A previous study showed that MDVs were generated rapidly under mild oxidative stress to protect cardiomyocytes (Li et al., 2020). Stress-induced MDVs are a pathway to remove oxidized proteins or lipids from mitochondria (Soubannier et al., 2012).



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Fig. 2. CO preconditioning induces massive generation of MDVs in OPCs. (a) OPCs were stained with PDH (Red), TOM20 (Green), and Hoechst (Blue) in control, 2 h- and 5 h- 2.5 μ M CORM-2 treated groups. MDVs were either PDH positive or TOM20 positive marked with white arrows. Scale bar = 5 μ m. (b) Quantification of MDVs was manually calculated in the above three groups, $n = 22$ random cells per group from 3 independent experiments (DF=2, $F=56.47$, $^{**}P < 0.01$). (c) Mitochondrial circularity didn't show any difference in the 2 h-treatment group but was significantly higher in the 5 h-treatment group than that in the control group (DF=2, $F=9.610$, $^{**}P < 0.01$) $n = 30$ –50 random cells per group of experiment. (d) The Red/green fluorescence intensity ratio was quantified at 1, 2, 3, 4, and 5 h in 2.5 μ M CORM-2 treated OPCs to compare with the non-treated control group. CCCP (50 μ Mol, 10 min) was applied as the positive control (DF=6, $F=18.67$, $^{**}P < 0.01$), $n = 30$ cells per group from 3 independent experiments. (e) The JC-1 staining images were shown in control, CORM-2 (2 h), and CCCP (10 min) treatment groups. Scale bar = 20 μ m. Statistical analysis was done using one-way ANOVA.

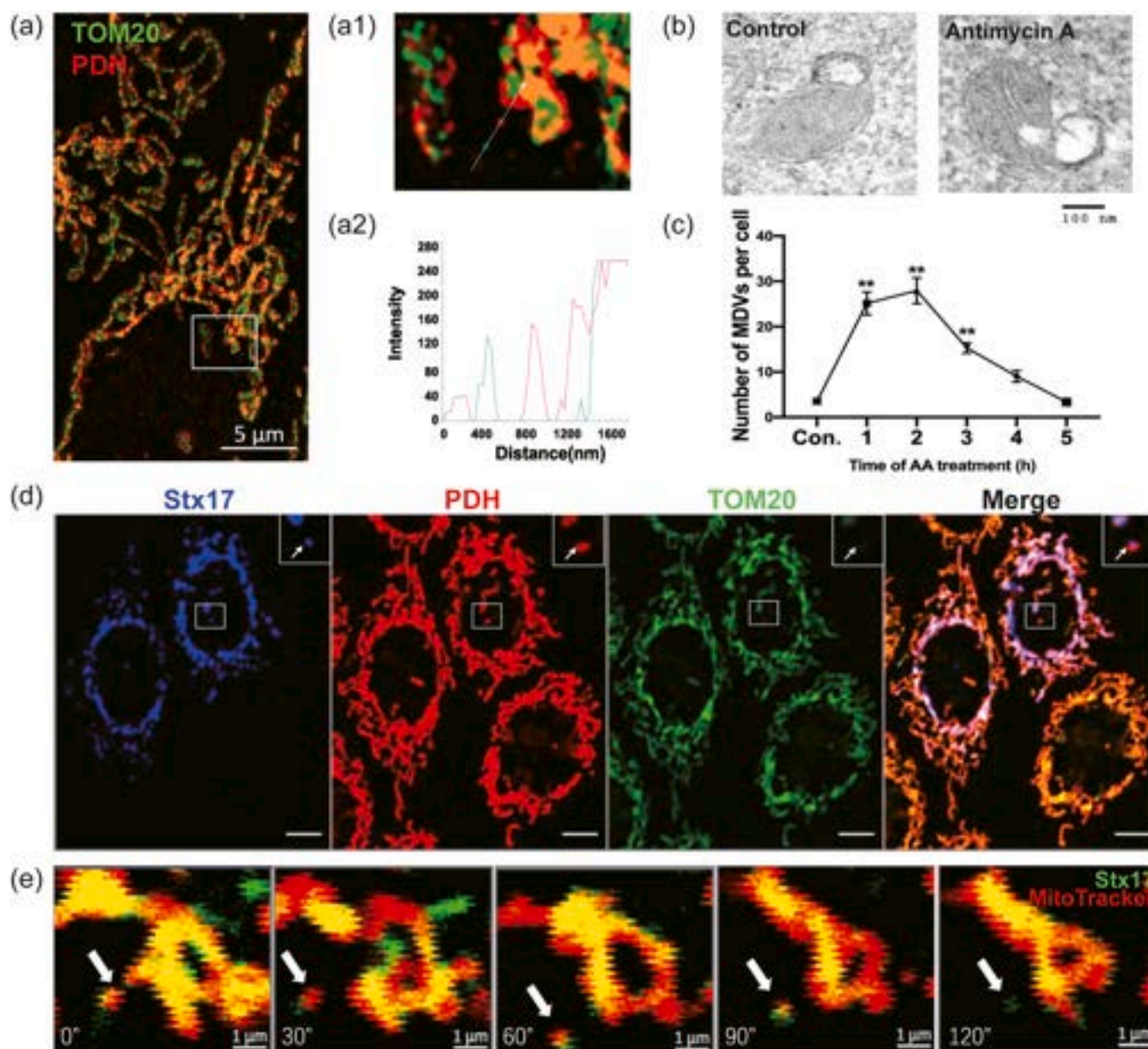


Fig. 3. MDVs can be stimulated by antimycin A and transported by stx17 in CHO cells. (a) Antimycin A (10 μ M, 2h) treated CHO cells were stained with TOM20 (green) and PDH (red), and observed by super-resolution structured illumination microscopy (SIM). (a1-a2) The fluorescent intensity of the green or red signal was shown by an intensity plot of the line drawn in the enlarged area. Scale bar = 5 μ m. (b) MDVs with a diameter of 70–150 nm were observed in both control and AA-treated cells by EM. Scale bar = 100 nm. (c) MDVs were quantified in CHO cells treated with antimycin A for up to 5 h under Z2 microscope (DF=5, $F=37.31$, $^{**}p < 0.01$), $n = 30$ random cells per group from 3 independent experiments. (d) YFP-Stx-17 (cyan) transfected CHO cells were labeled with antibodies against PDH (red) and TOM20 (green). The enlarged area showed colocalization of Stx17 and PDH but not TOM20. Scale bar = 5 μ m. (e) Live-cell imaging showed MDVs with YFP-stx17 (green) and Mito Tracker (red) were degraded within 120 s. Scale bar = 1 μ m.

The present study found that a low dose of CORM-2 could induce massive generation of MDVs. This agrees with previous studies that MDVs could be stimulated by several forms of oxidative stress. Antimycin A, a complex III inhibitor, is the most frequently tested inducer of

MDVs. Xanthine/xanthine oxidase and doxorubicin, two sources of H_2O_2 , promote a robust increase in the production of both TOM20⁺ and PDH⁺ MDVs in H9c2 myoblasts (Cadete et al., 2016). Glucose oxidase, an reactive oxygen species (ROS)-generating enzyme, can robustly

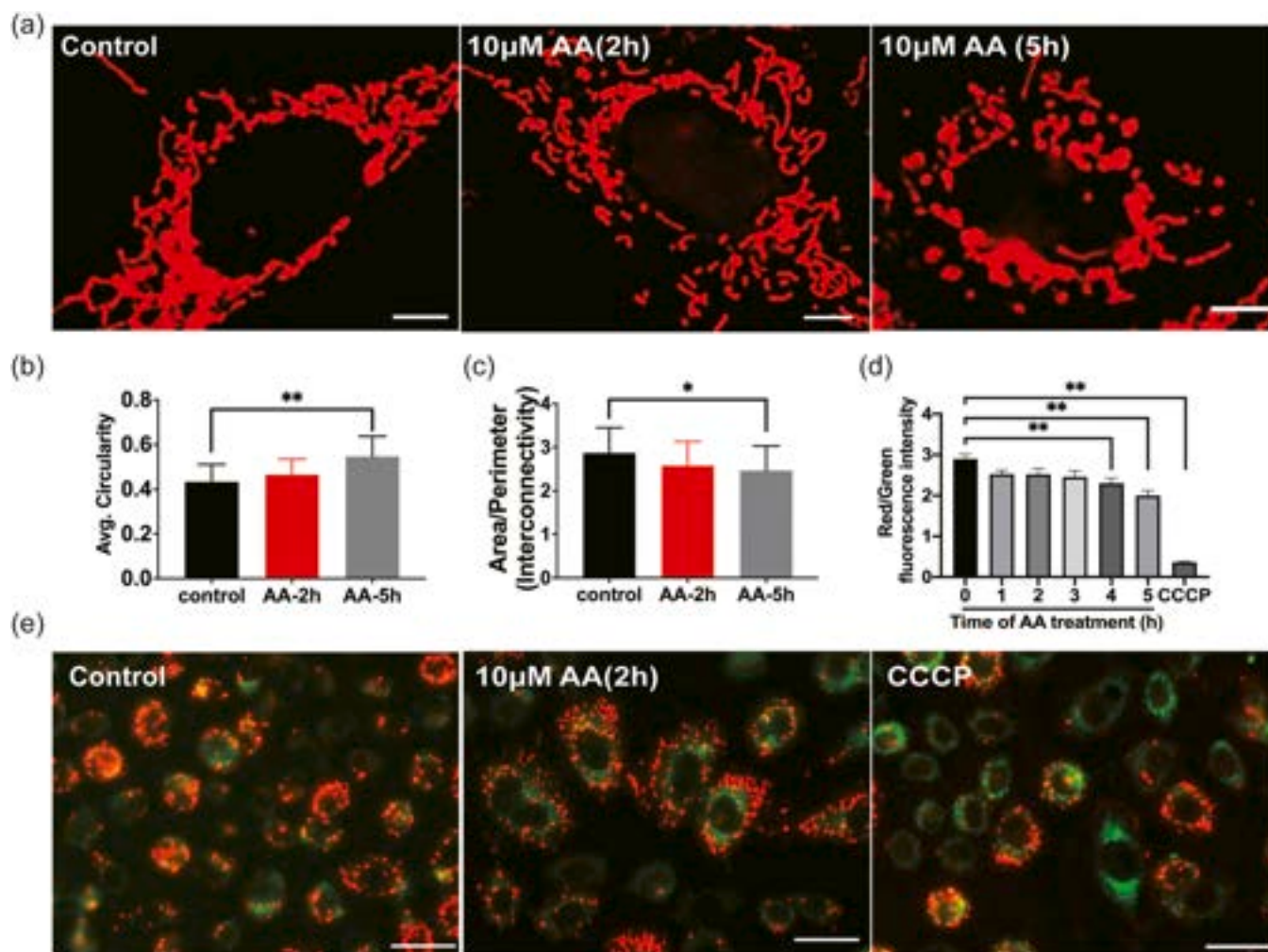


Fig. 4. MDV formation causes no detectable morphological changes or depolarization in CHO cells. (a) Mitochondria stained with MitoTracker Red didn't show significant changes in the 10 μ M AA-2 h group compared with the control group but became rounded and fragmented in the 5 h group. Scale bar = 5 μ m. (b-c) The index of circularity was increased (DF=2, F=17.00, ** $p < 0.01$), but mitochondrial interconnectivity (Area/ Perimeter) was decreased dramatically (DF=2, F=4.173, * $p < 0.05$) in 10 μ M AA-5 h group. However, there was no significant change in 10 μ M AA-2 h group, vs. control group, $n = 31$ random cells per group from 3 independent experiment. (d) Quantification of mitochondrial membrane potential by fluorescence intensity of red/green ratio using JC-1 test. CHO cells were treated with AA from 1 to 5 h to compare with control cells. CCCP (50 μ M, 10 min) was applied as positive control (DF=6, F=49.55, ** $P < 0.01$). $n = 60$ random cells per group from 3 independent experiment. (e) CHO cells were stained with JC-1 probe in control, AA (2 h), and CCCP groups. Scale bar = 20 μ m.

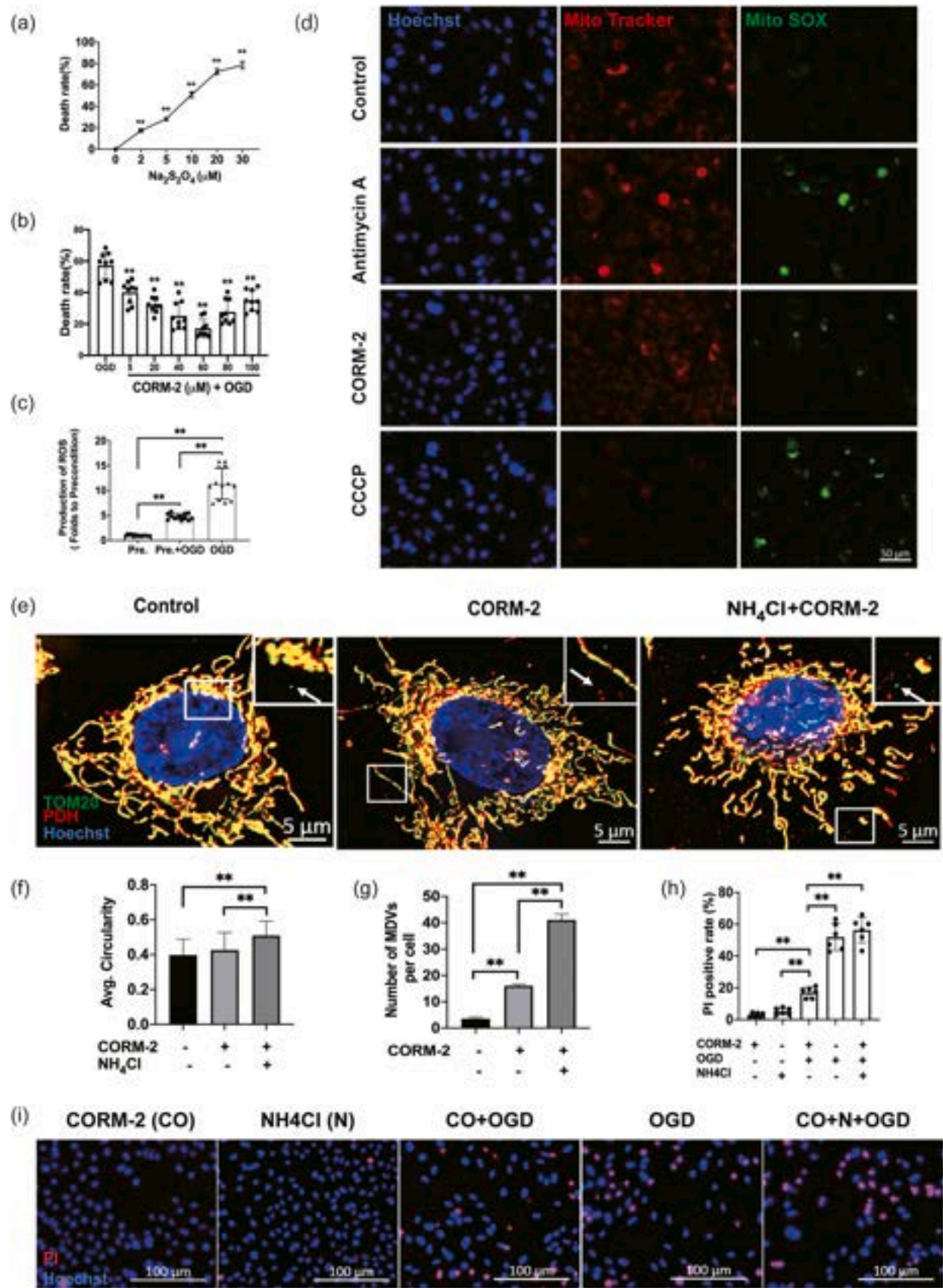
generate MDVs within 90 min at a subtoxic dose in COS7 cells (Soubannier et al., 2012). Heat stress at 42 $^{\circ}$ C for 45-min generates massive MDVs in macrophage cells (Wang et al., 2016).

A previous study shows that Stx17, a SNARE protein associated with vesicle fusion, is a transporter of MDVs to fuse with lysosomes (McLellan et al., 2016). In our study, we observed that stx17 partially colocalizes with PDH-positive MDVs. There are still some MDVs (either PDH or TOM20 positive) that are not colocalized with stx17, indicating that stx17 may participate in the transportation of some but not all MDVs. Live-cell imaging on YFP-Stx17 transfected CHO cells showed that MDVs were generated from mitochondria and degraded within 120 s. Lysosome is one of the destinations for MDVs (Soubannier et al., 2012). Transportation of cargo-specific MDV is arrested upon abrogation of the endolysosomal pathway (Soubannier et al., 2012). Our data show MDVs are accumulated in the cytosol when lysosome activity is inhibited by NH₄Cl, leading to more circulated mitochondria with less interconnectivity. It is indicated that the protective effect of preconditioning was counteracted by lysosome inhibition. All those results strongly support that the protective mechanism of CO preconditioning involves the activation of MDV-mediated mitochondrial quality control.

Mitochondria are the central target of preconditioning and

postconditioning strategies (Garcia-Nino et al., 2021). In this study, we found that mitochondrial morphology and membrane potential in OPCs didn't change significantly under CO-preconditioning induced by 2.5 μ M CORM-2 for 2 h but became highly circular and less connected after 5 h. TOM20 positive and PDH positive MDVs were found adjacent to mitochondria in OPCs when treated with CORM-2 for 2 h, but MDVs were rarely generated after 5 h. Our results suggest that MDVs are generated rapidly under mild oxidative stress. Previous studies also support that generation of mitochondrial-derived vesicles is an earlier response to oxidative stress (Matheoud et al., 2016) to remove oxidized proteins or lipids from mitochondria (Soubannier et al., 2012).

Carbon monoxide at high doses leads to fatal poisoning immediately or delayed neurologic sequelae (DNS) within days to weeks (Sekiya et al., 2019). In the brain, oligodendrocytes are particularly susceptible to CO toxicity, which is considered a contributing factor to demyelination in DNS (Tian et al., 2020). In the study, we found CO-preconditioning by a low concentration of CORM-2 could protect OPCs from apoptosis through inhibiting AIF translocation from mitochondria to the nucleus. Although further studies are needed to clarify pathways of OPC death, CO-preconditioning appears to be a promising strategy for OPC protection.



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Fig. 5. CO preconditioning stimulated MDV formation through generation of reactive oxygen species in CHO cells. (a) Quantification of the death rate under OGD condition induced by different concentrations of $\text{Na}_2\text{S}_2\text{O}_4$ for 3 h in CHO cells. IC_{50} of $\text{Na}_2\text{S}_2\text{O}_4$ was 11.38 mM, $n = 3$ independent cell culture preparations (DF=5, $F=209.4$, $**p < 0.01$), $n = 3$ independent experiments. (b) Quantification of cell death rate in CHO cells when they were preconditioned with various concentrations of CORM-2 before OGD (DF=6, $F=28.43$, $**p < 0.01$), $n = 3$ independent experiments. (c) Quantification of cellular ROS production in preconditioned with 60 μM CORM-2 for 2 h (Pre.), Pre + OGD, and OGD groups, vs. control (DF=2, $F=105.8$, $**p < 0.01$), $n = 3$ independent experiments. (d) Levels of ROS in mitochondria were detected with Mito SOX assay in CHO cells treated with buffer only (Control), antimycin A (10 μM , 2 h), CORM-2 (60 μM , 2 h), or CCCP (50 μM , 10 min). Scale bar = 100 μm . (e) CHO cells were stained with TOM20 (green), PDH (red), and Hoechst (blue) in control, 60 μM CORM-2 (2 h) and 60 μM CORM-2 + 10 μM NH_4Cl (2 h) treated groups. Boxes highlight MDVs with either PDH or TOM20-positive staining. Scale bar = 5 μm . (f) Mitochondrial average circularity was analyzed by Image J software to compare control, CORM-2, and NH_4Cl +CORM-2 treated groups (DF=2, $F=19.93$, $**p < 0.01$), $n = 3$ independent experiments. (g) Number of MDVs was calculated manually in the above three groups of cells (DF=2, $F=189.3$, $**p < 0.01$), $n = 30$ –50 random cells per group from 3 independent experiments. (h–i) The CHO cells were stained with PI (Red) and Hoechst (Blue). The PI positive cells were quantified in CORM-2 (CO), NH_4Cl (N), OGD(O) and CORM-2 + NH_4Cl + OGD (CO+N + O) groups compared with CORM-2 + OGD (Preconditioning) group (DF=4, $F=129.3$, $**p < 0.01$), $n = 3$ independent experiments. Scale bar = 100 μm .

In summary, CO preconditioning activates massive production of MDVs and ameliorates oxidative damage to cells. MDV-mediated mitochondrial quality control is a mechanism underlying CO-preconditioning.

Ethical Approval and Consent to participate

All our work was reviewed and approved by the Animal Care and Use Subcommittee at the University of Manitoba.

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CRediT authorship contribution statement

Y. G. and J. K. designed the project. J. K. and G. Z. supervised the study. Y. G., T. G., X. J., and X. T. performed the experiments and acquired the data. Y. G. and X. J. produced the figures and carried out the statistical analysis. Y. G. drafted manuscript text. T.G and C. J. edited the manuscript. J. K. and G. Z. critically reviewed and revised the manuscript. All authors read and approved the manuscript.

Data Availability

Data will be made available on request.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Consent for publication

The authors consent to publishing this work.

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