The Secret Life of Mitochondria

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5 General discussion

Preface

Mitochondria are dynamic organelles, not only functioning inside host cells, but also actively transferred to surrounding cells, facilitating intercellular communication and coordination. Mitochondria can be transferred between cells through both direct and indirect mechanisms. In the absence of physical contact, cells can release free mitochondria and/or mitochondrial-containing extracellular vesicles (mitoEVs). Alternatively, cells can also form tubular structures between each other that traffic mitochondria. Over the past decade, an emerging body of research has uncovered that intercellular mitochondrial transfer (MT) plays a pivotal role in normal physiological functions and has been implicated in the pathogenesis of numerous diseases. Broadly, the implication of MT can be categorized into two domains: its potential as a therapeutic intervention and its utility as a biomarker. Despite these promising applications, the role of MT within the context of neuroscience remains insufficiently explored. This thesis seeks to address this critical gap by investigating the involvement of extracellular mitochondria in three interconnected aspects: i) the neuroprotective potential; ii) the possibility to use them as biomarkers; and iii) the role in mediating interactions between brain-resident cells and tumor cells.

Challenges in preventing neurodegeneration

Neurodegeneration is a hallmark of various brain disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS)[1]. Patients with these neurodegenerative diseases experience significant physical and psychological burden, frequently resulting in a progressive loss of functional independence and quality of life. Consequently, intensive health care investment is required, placing a substantial burden on both society and their families. To date, no effective approaches have been developed for preventing or treating neurodegeneration. This is primarily due to the complexity of the pathophysiology and heterogeneity of these disease symptoms. Moreover, lack of early diagnostic tools and treatment options further complicates efforts to combat these conditions, as symptoms typically manifest only after significant neuronal loss has occurred. Therefore, the development of reliable biomarkers for early diagnosis, along with universally applicable treatment strategies, is urgently needed.

The common mechanistic factors behind neurodegenerative disorders involve protein misfolding and aggregation, oxidative stress, neuroinflammation, and

mitochondrial dysfunction. Among these, mitochondria stand as the key element for those mechanisms as they are interplaying with reactive oxygen species (ROS) generation and the regulation of neuroinflammation[2]. Mitochondrial dysfunction is recognized as one of the earliest events in neurodegeneration and occurs throughout its progression, making it a promising target for monitoring and intervention[3]. Consequently, the field of mitochondrial medicine and diagnostics has garnered increasing attention in recent years.

Part I. Neuroprotective potential: mitochondrial transplantation in ferroptosis

Typical mitochondrial medicine involves supplementation of enzymatic cofactors, antioxidants, and other essential nutrients[4]. Nevertheless, those supplements can result in multiple side effects. For instance, high doses of antioxidants will not only capture detrimental ROS[5] but also inactivate the ETC[6]. More recently, gene therapy has emerged as a promising approach to address genetic mutations associated with mitochondrial diseases. Advances in this field include several treatments, for example utilizing mRNA or adeno-associated virus (AAV) delivery has shown significant improvements in the mouse model of Leber hereditary optic neuropathy (LHON) and are currently under clinical trials[7, 8]. Due the heterogeneity of mitochondrial-related diseases, personalized medicine designed for specific mitochondrial deficiency is needed. However, this necessity significantly complicates the development and broad application of treatments across diverse patient populations.

Since the initial observation in 1982 that purified mitochondria could enter co-cultured cells[9], researchers have extensively explored the potential of applying free mitochondria in different conditions. In the field of neuroscience, an important study uncovered the phenomenon that healthy astrocytes release free mitochondria which can enter ischemic neurons and protect them from cell death[10]. Thereafter, a novel therapeutic method termed mitochondrial transplantation has been introduced to treat neurodegeneration. Basically, this method is to freshly isolate healthy mitochondria from cell lines or tissue, followed by applying them directly to affected cells, tissue or individuals. Up to date, mitochondrial transplantation has been widely reported to show protective effects in different disease models, including aging[11], PD[12], and AD[13]. Furthermore, no immunological response was observed in these studies, suggesting that healthy

mitochondria—whether autologous, allogeneic, or xenogeneic—do not induce transplant rejection, either in vitro or in vivo.

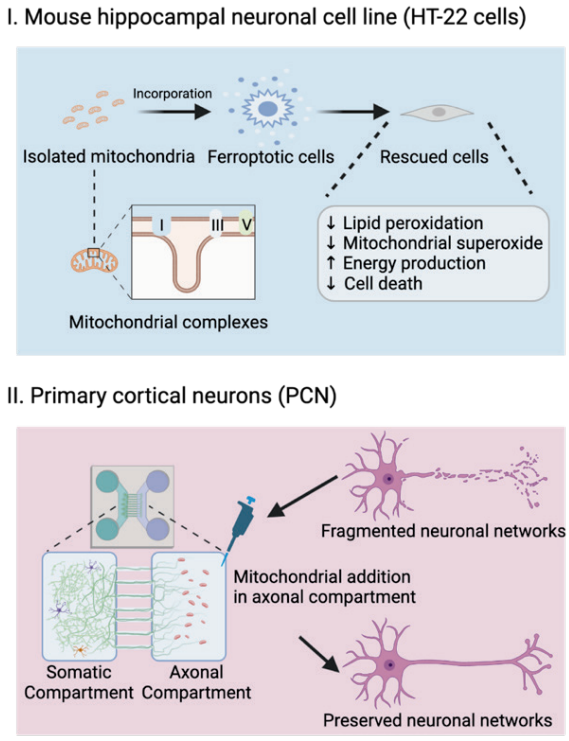

Figure 1. Graphical abstract of chapter 1.

Our data show that I) exogenous mitochondria can be incorporated into mouse hippocampal neuronal cell line, HT-22 cells. The incorporation protects host cells against RSL3-induced ferroptosis. ETC components, mitochondrial complex I, III, and V were identified to participate this neuroprotective effect, which results in decreased oxidative stress, increased ATP production, and eventually attenuated cell death. II) Seeding PCN on the somatic compartment of a microfluidic device allowing us to separate somata from extended neuronal network when PCN reach mature stage. Treating RSL3 in somatic compartment induces ferroptotic damage in PCN, e.g., fragment neuronal networks. Applying mitochondria in axonal compartment leads to their transportation to somatic compartment which is verified to preserve the damaged neuronal networks. The drawing was created using BioRender.com.

Since the common feature of neurodegeneration is progressive loss of neurons in the central nervous system (CNS), the primary goal of using mitochondrial transplantation in the prevention and rescue of neurodegeneration is to prevent neurons from cell death. Previous studies showed transplanting healthy mitochondria significantly alleviated apoptotic death[12, 14]. However, there is currently no evidence showing its effectiveness on ferroptosis, a recently identified form of cell death that play a crucial role in the onset and progression of neurodegenerative

disorders[15]. In **Chapter 2,** we apply mitochondrial transplantation for preventing neuronal ferroptosis. The results show the internalization of exogenous mitochondria in neurons that further increase cell viability, alleviate cell death, and restore neuronal networks (Figure 1).

To advance this research toward future clinical translation, we have outlined the following technical challenges/perspectives for future research.

1. How to preserve/boost the function of isolated mitochondria?

Preserving the function of isolated mitochondria is essential for their protective effects, as damaged mitochondria can exacerbate cell death (data not shown) and OXPHOS inhibitors applied on mitochondria could lead to loss of their protective effects (**Chapter 2, figure 4D-E**). To address this, the preparation of isolated mitochondria in this study was meticulously conducted to ensure their integrity and functionality. First, a specialized isolation buffer containing several key components was used: hydroxyethyl piperazine ethane sulfonic acid (HEPES) for retaining physiological pH (7.2-7.4); ethylenediaminetetraacetic acid (EDTA) for chelating excessive divalent cations (like calcium and magnesium); and sucrose for stabilizing the osmotic stress. Moreover, all procedures were strictly performed at 4°C to stabilize enzymatic activity, minimize ROS production, and prevent membrane degradation. The entire isolation procedure was conducted swiftly and with precision. Mitochondria were immediately applied on cells to ensure that mitochondria were applied to cells within a few hours of preparation. Up to date, long-term storage of isolated mitochondria remains unfeasible, presenting a significant challenge to the clinical translation of mitochondrial transplantation.

Therefore, increase their efficiency in entering host cells and subsequently preserving the functionality of exogenous mitochondria are critical for the successful application of mitochondrial transplantation.

The main strategies to achieve these goals can be summarized into the following key approaches.

1) Mitochondrial surface modification. Cell membrane penetrating proteins, such as Pep-1 and TAT, which has shown to effectively prompt the internalization of foreign mitochondria in host cells, and validate in several disease, including lactic acidosis, stroke-like episodes (MELAS), PD and perfusion injuries[12, 16].

2) Targeting membrane permeability of host cells. Another study combined centrifugal force and reagents for increasing the fluidity of cell membranes,

which largely increased the entry of mitochondria in host cells and enhanced cell metabolic function[17]. Moreover, pre-coating exogenous mitochondria with TPP^+ (a triphenylphosphonium cation compound) and dextran complexes can reduce the high negative charge of their encapsulated mitochondria and mitigate the electrostatic repulsion between cell membrane and exogenous mitochondria. These characteristics enable high delivery efficiency and selective power for high-quality mitochondria, ensuring the functional integrity of the transplanted organelles[18].

3) Delivery via carriers. Patel et al. developed a thermal-gelling and erodible hydrogel system fabricated with methylcellulose and hyaluronic acid to encapsulate mitochondria. This innovative approach was demonstrated to effectively preserve the respiratory capacity of the encapsulated mitochondria[19]. Another substance Pluronic F127 (PF127) hydrogel showed similar effect[20].

4) Delivery via lipid membranes. Researchers developed membrane structures, either artificial lipid membrane or naturally cell-derived extracellular vesicles, being shown to effectively deliver functional mitochondria[21, 22].

5) Other novel strategies. Other methods, such as magnet-driven techniques, photothermal nano-blades, and automation-based micro-manipulation using optical tweezers, have emerged as promising delivery strategies for enhancing the delivery efficiency and precision of mitochondrial transplantation[23].

2. Are the entire mitochondria needed for observed protective effects?

Current sources of mitochondria for transplantation are mostly represented by isolation of mitochondria from fresh tissue or cells. This dependency imposes significant limitations on mitochondrial availability and poses challenges for preserving their functionality over time, thereby hindering their clinical application. To address these challenges, synthetic biology methods have been introduced to reconstruct “artificial mitochondria” *in vitro*. Researchers attempt to make a respiratory nanoreactor to regenerate ATP and the triggers can be detergent, light, or chemicals. The system established is based on a biomimetic system surrounded by membrane structures which can produce ATP, through specific triggers, such as detergent, light, or chemicals[24-26]. Moreover, the catechol metal ions produced FEx-1 nanoreactor and can produce energy and also reduce ROS generation[26]. Biosynthetic “artificial mitochondria” provide a controllable and customizable tool for advancing the application of mitochondrial transplantation, addressing challenges related to availability, functionality, and preservation.

3. Do transplanted mitochondria elicit any functions in acceptor cells?

Understanding the mechanism behind neuroprotective effects of mitochondrial transplantation can provide critical insights into optimization of targeted therapies. For instance, delivering only the effective components of exogenous mitochondria to disease model can enhance the therapeutic efficacy and can potentially simplify the storage condition. We preformed several experiments in **chapter 2** to address this question. First, we observed that exogenous mitochondria significantly decreased lipid peroxidation and mitochondrial superoxide in ferroptotic acceptor HT22 cells, indicating that the transplanted mitochondria could alleviate ferroptotic pathways (**Chapter 2, figure 3**). This is potentially due to the effects mediated by the application of exogenous mitochondria on antioxidant redox balance, iron metabolism, mitochondrial activity and/or lipid remodeling. It is well-known that mitochondria exhibit robust antioxidant effects, which are attributed to i) enzymatic antioxidants such as glutathione peroxidase (GPX) and peroxiredoxins, catalase; and to ii) non-enzymatic antioxidants such as glutathione (GSH), coenzyme Q10 (CoQ10), and vitamin E[27]. Moreover, mitochondria also regulate iron metabolism, as they contain iron storage (mitochondrial ferritin) and transport (mitoferrin-1 and -2) proteins[28]. We also demonstrated that exogenous mitochondria significantly increase oxygen consumption capacity in acceptor cells (**Chapter 2, figure 4A**), and the enhanced bioenergetics can interplay with cellular signaling pathways, metabolic adaptations, and stress responses.

From the perspective of a protective provider, through using specific inhibitors of mitochondrial OXPHOS, we identified that complex I, III, and V were vital elements contributing to the protective effects of exogenous mitochondria (**Chapter 2, figure 4B-E**). However, utilizing those inhibitors did not fully block the effect of exogenous mitochondria against ferroptosis. One possible reason is that they did not achieve their full inhibitory capacity. Nevertheless, mitochondrial OXPHOS is important and should considered as an essential component in the therapeutic cargo of mitochondrial transplantation treatment.

Part II. Biomarkers: investigating protein contents in mitoEVs of familial AD model

Another way of mitochondrial transfer is through lipid bilayer structures—EVs. Previous studies have demonstrated that EVs, from diverse sample types, including patient cerebrospinal fluid (CSF), blood, post-mortem brain tissue, and cell models, play a critical role in the delivery and propagation of neurotoxic proteins that induce AD[29-32]. EVs are released by nearly all brain cell types, with neurons being one of the most significant contributors. Tian et al., compared the protein cargo of plasma EVs from AD patients, PD patients, as well as age-matched healthy controls. Their study identified the neuron-derived synaptic protein NMDAR2A in peripheral blood-derived EVs as a potential biomarker for AD[31]. Moreover, cell lines with AD mutations secrete EVs contain soluble APP (sAPP) protein β, sAPPα, and soluble Aβ1–42[33, 34]. These studies emphasize the significant role of neuron derived EVs in the progression and pathology of AD. However, no studies have explored the identification of biomarkers within EVs derived from early stages of neuronal development, which could serve as a more effective window for implementing therapeutic interventions. Our study sought to investigate proteomic alterations of EVs derived from NPCs with *PSEN1ΔE9* mutation, a model for studying early phases of familial AD neurogenesis. In **chapter 3**, we identified the proteomic alterations of EVs from neural progenitor cells (NPCs) carrying *PSEN1ΔE9* mutation (workflow illustrated in figure 2). Our key findings are:

I. *PSEN1 ΔE9* mutation does not alter the size and yield of EVs derived from NPCs.

II. Significantly decreased protein complexity is observed in AD EVs compared to isogenic counterparts.

III. Several key proteins, such as pregnancy zone protein (PZP), alkaline phosphatase (ALPL), periostin (POTSN), apolipoprotein c3 (APOC3), and transferrin (TF), were identified for the candidates of AD biomarkers.

IV. The accumulation of mitochondrial matrix proteins in AD EVs could be potential indication of impaired energy properties in AD NPCs.

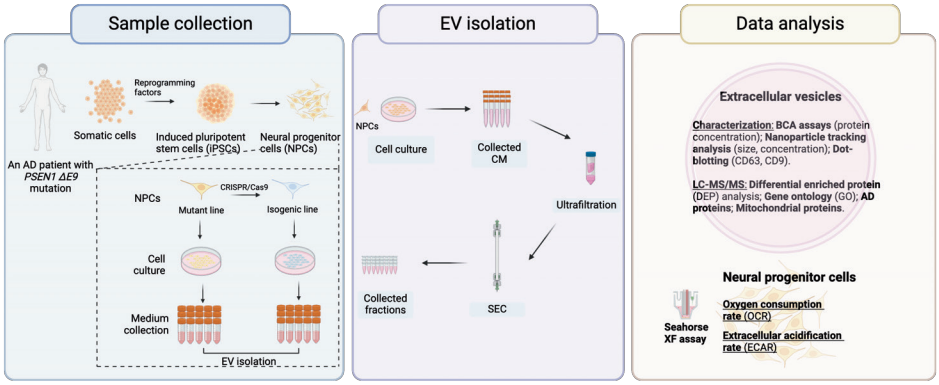

Figure 2. Workflow in chapter 3.

Briefly, the workflow of chapter 3 contains three main sections. For the sample collection, neural progenitor cells (NPCs) were originally generated from an AD patient harboring *PSEN1 ΔE9* mutation. The gene corrected isogenic in the iPSCs was used as a control. Through culturing these two cell lines, conditioned medium (CM) was collected for EVs isolation. The collected CM were then concentrated through ultrafiltration. Using size exclusion chromatography (SEC), a total of 24 fractions were collected. For the data analysis, we firstly performed BCA assays and dot-blotting, by which we identified EVs fractions out of 24 collect fractions. Nanoparticle tracking analysis (NTA) was performed to assess the size and concentration of EV fractions. Followed by LC-MS/MS, we conducted differential enriched protein (DEP) analysis and function annotations such as gene ontology (GO), AD and mitochondrial protein identification. To correlate our findings in proteomics, we performed seahorse XF assays to check mitochondrial respiration and glycolysis. The illustration is made with Biorender.com.

Proteomic alterations and AD pathology

Using LC-MS/MS, we identified 20 proteins associated with AD pathology that are upregulated in AD EVs (**Chapter 3, figure 5**). These proteins are involved in key biological functions, including ECM organization—such as the regulation of fibrinolysis and collagen fibril organization—as well as lipid metabolic processes and the regulation of phagocytosis. ECM remodeling is the prominent biological process regulated by proteins present in AD EVs, as 4 out of 20 proteins are intensively involved in this pathway. Fibrinolysis plays a crucial role in the brain's ECM function, particularly under pathological conditions. Our study identified two proteins SERPINF2 and PLG that are involved in fibrinolysis. SERPINF2, also known as alpha 2-antiplasmin (or α2-antiplasmin or plasmin inhibitor), is a serine protease inhibitor (serpin) responsible for inactivating plasmin. Plasmin has been shown to play a dual role in AD pathology: it facilitates Aβ clearance, reducing amyloid burden, but also contributes to ECM degradation, which can compromise neuronal structure and function[35, 36]. SERPINF2 inhibits plasmin activity thereby maintains the structural integrity of collagen fibrils. Interestingly,

the inactive precursor of plasmin—plasminogen (PLG), is also upregulated in AD EVs. Collectively, our findings demonstrate that EV-mediated transport potentially influence both protective and detrimental pathways in AD. Future studies could focus on evaluating the expression levels and enzymatic activity of plasmin, as well as analyzing the composition and dynamics of the ECM, to gain deeper insights into the underlying mechanisms and their contributions to AD pathology.

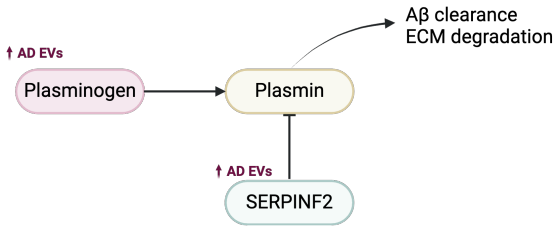

Figure 3. The function of upregulated fibrinolysis proteins in AD EVs.

The illustration is made with Biorender.com.

Proteomic alterations and mitochondrial function

Our study identified mitochondrial proteins in EVs derived from NPCs. Notably, some of them found to be upregulated in AD EVs within our dataset, including Isocitrate dehydrogenase 2 (IDH2), aldehyde dehydrogenase 2 (ALDH2), carbamoyl-phosphate synthase 1 (CPS1), transcarbamylase (OTC), propionyl-CoA carboxylase beta subunit (PCCB), aldehyde dehydrogenase 1 family member L1 (ALDH1L1), and aldehyde dehydrogenase 9 family member A1 (ALDH9A1) (**Chapter 3, figure 6B**). Meanwhile, impaired energy profiles, including decreased mitochondrial respiration and glycolysis, were observed in the correspondent AD NPCs (**Chapter 3, figure 7**).

Among significantly enriched mitochondrial proteins in AD EVs, IDH2, ALDH2, CPS1, OTC, and PCCB are closely related to mitochondrial respiration. IDH2 participates in the TCA cycle and catalyzes the conversion of isocitrate to α-ketoglutarate and produce NADPH in this process. ALDH2 supports the TCA cycle by metabolizing intermediates such as acetaldehyde. PCCB participates in the catabolism of odd-chain fatty acids and certain amino acids, converting propionyl-CoA to methylmalonyl-CoA. This supplies intermediates for the TCA cycle, linking amino acid and lipid metabolism to mitochondrial energy production. While CSP1 and ornithine OTC primarily function in urea cycle but not indirectly participate the TCA cycle by maintaining metabolic balance. Similarly, ALDH1L1 mediates folate metabolism, and it has been implicated in mitochondrial metabolism through affecting 5-aminoimidazole-4-carboxamide ribonucleotide

accumulation and serine depletion[37]. ALDH9A1 catalyzes the dehydrogenation of gamma-aminobutyraldehyde (GABAL) to gamma-aminobutyric acid (GABA), indirectly influencing glycolysis and overall energy metabolism.

We observed that the identified mitochondrial proteins in AD EVs are directly or indirectly associated with OXPHOS or glycolysis, which explains the energetic deficits observed in AD NPCs. This suggests that the delivery of mitochondrial matrix proteins through EVs reflects the impaired mitochondrial health in donor cells. Alternatively, mitoEVs may also represent a compensatory mechanism to support surrounding cells through delivering functional proteins. Future research is necessary for validating the biological function of mitoEVs in recipient/acceptor cells to better understand their role in AD pathophysiology.

Part III. Tumorigenesis: GBM cells hijack neuronal mitochondrial through tubular-like structures

In addition to secretion pathways, mitochondria can also be transferred through direct formation of tubular structures that connect two cells. The transfer of mitochondria via tubular structures can lead to various outcomes depending on the context and the physiological states of the donor and recipient cells. For protective perspectives, healthy cells transfer functional mitochondria to stressed or damaged cells with impaired mitochondrial function[38]. Under pathological conditions, damaged or dysfunctional mitochondria are exported from stressed cells to healthy neighboring cells for degradation or spreading the damage[39]. Notably, this type of transfer can also occur bidirectionally for balancing mitochondrial populations and functional capacities[40]. Therefore, the transfer of mitochondria through tubular structures is a double-edged sword. Understanding these dynamics is vital for leveraging MT in therapeutic strategies.

Mitochondria-containing tubular structures have been identified in the brain where they function in neurodegeneration and facilitate glioblastoma (GBM) progression. α-synuclein (α-syn, the neurotoxic aggregates that cause PD) overload microglia form tunneling nanotubes (TNTs) with healthy microglia, allowing the disposal and degradation of α-syn. With the same structure, healthy microglia donate mitochondria to α-synuclein overloaded cells, aiding in their recovery and supporting mitochondrial function[41]. Similarly, α-syn and tau (hallmarks of AD) overburden neurons receive healthy mitochondria from surrounding microglia,

resulting in alleviated oxidative stress, normalized gene expression, and cell survival[42, 43]. In the context of brain tumor, mitochondria are observed to be transferred through tubular structures from astrocytes and microglia to GBM cells. The acquisition of astrocyte mitochondria in GBM leads to increased mitochondrial respiration, upregulated metabolic pathways for proliferation and tumorigenicity, and enhanced proliferative phenotype in GBM cells[44].

In **chapter 4**, we aimed to understand how GBM cells survive in the brain by examining their interconnection with neurons through tubular structures. We found tubes formed between neurons and GBM cells, allowing the occurrence of massive MT from neurons to GBM cells. These mitochondria increased energy metabolism and cell proliferation in GBM cells. These findings underscore the critical role of neuronal connections in GBM progression and suggest mitochondrial transfer pathways as promising therapeutic targets for cancer.

Limitations and future perspectives

1. Are the observed effects associated with transferred mitochondria?

We observed MT from neurons to GBM cells and confirmed this transfer predominantly relies on physical contact. Subsequently, we employed cell sorting for selecting GBM cells containing neuronal mitochondria and assessed their functional alterations compared to monocultured GBM cells. However, no direct evidence attributes these effects to the transferred mitochondria, as other bioactive substance can also cause similar effect, such as calcium[45]. Future steps can try extracting mitochondria from neurons to directly confirm their role in these functional changes.

2. Assessing the importance of tubular structures in mediating mitochondria transfer.

Previous studies have conducted through either pharmacological intervention, like cytochalasin B (inhibition of actin) or vincristine (inhibition of microtubule), or gene modifications, such as knocking down GAP43 expression, to address the question that whether tubular structures are essential condition for transferring mitochondria. However, we were unable to employ these tools due to technical limitations and the fragile nature of neuronal cultures, which do not survive the procedures required for such techniques. To address this challenge, we attempted to improve tube formation by applying TGFβ, a factor known to promote tumor microtube and neuronal network formation[46, 47]. We observed a trend toward increased mitochondrial transfer in GBM cells following the application of TGFβ;

however, this change was not statistically significant, likely due to high efficiency of MT occurring in the overnight incubation. Future approaches could involve reducing the incubation and testing other inhibitors without compromising cellular survival.

3. More functional assessments.

We examined the functional outcomes of MT in GBM cells and found that neuronal mitochondria could increase both bioenergetic and proliferative patterns. However, for tumor progression, it is crucial to determine whether MT also influences invasiveness and resistance to therapies, which can be explored in future experiments.

Summary of key findings in this thesis

Collectively, this study explored three distinct mechanisms of mitochondria transfer, free mitochondria (mitochondrial transplantation), mitochondria containing EVs (mitoEVs), and tubular structures. Our findings highlight the critical roles of these mechanisms could play in cell-to-cell communication. Specifically, we demonstrated the neuroprotective effects of mitochondrial transplantation in alleviating ferroptosis, the role of mitoEVs in delivering signals in the early stage of AD progression, and the contribution of tubular structures in supporting tumor growth. These insights pave the way for further exploration into therapeutic strategies targeting mitochondrial transfer to modulate neurodegeneration and brain cancer progression.

Main conclusions

1. Exogenous healthy mitochondria demonstrate a robust neuroprotective effect by incorporating into recipient cells and mitigating ferroptosis. In HT-22 cells, mitochondrial transplantation rescues cells from ferroptosis by attenuating lipid peroxidation and reducing mitochondrial superoxide production. The neuroprotective activity is mediated through enhanced activity of mitochondrial complexes I, III, and V. Additionally, exogenous mitochondria integrate into neuronal networks in primary cortical neurons (PCN), preventing network fragmentation and protecting against ferroptotic cell death (Chapter 2).

2. The PSEN1 ΔE9 mutation does not affect EV size or production in NPCs but reduces protein complexity in AD EVs. Key proteins (PZP, ALPL, POTSN, APOC3, TF) were identified as potential AD biomarkers, and mitochondrial matrix protein accumulation in AD EVs suggests impaired energy metabolism in NPCs (Chapter 3).

3. GBM cells hijack mitochondria from surrounding neurons to fuel their energetic and proliferative demand. This mitochondrial transport process relies on physical contact and is facilitated by tubular structures composed of F-actin, β-III tubulin, GAP43, and membrane proteins (Chapter 4).

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