Liu *et al. Journal of Translational Medicine* (2025) 23:568
https://doi.org/10.1186/s12967-025-06472-9

Journal of Translational Medicine

REVIEW Open Access

Therapeutic implications of mitochondrial transfer on stem cell fate in regenerative medicine

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Abstract

With the discovery of intercellular mitochondrial transfer, the intricate mitochondrial regulatory networks on stem cell fate have aroused intense academic interest. Apart from capturing freely released mitochondria from donor cells, stem cells are able to receive mitochondria through tunneling nanotubes (TNTs), gap junctional channels (GJCs) and extracellular vesicles (EVs), especially when undergoing stressful conditions such as inflammation, hypoxia, chemotherapy drug exposure, and irradiation. Stem cells that are potentiated by exogenous mitochondria show enhanced potential for proliferation, differentiation, and immunomodulation. The well-tolerated nature of either autogenous or allogenous mitochondria when locally injected in the human ischemic heart has validated the safety and therapeutic potential of mitochondrial transplantation. In children diagnosed with mitochondrial DNA deletion syndrome, functional improvements have been observed when empowering their hematopoietic stem cells with maternally derived mitochondria. Apart from the widely investigated applications of mitochondrial transfer in ischemia-reperfusion injury, neurodegenerative diseases and mitochondrial diseases etc., therapeutic potentials of mitochondrial transfer in tissue repair and regeneration are equally noteworthy, though there has been no systematic summary in this regard.

This review analyzed the research and development trends of mitochondrial transfer in stem cells and regenerative medicine over the past decade from a bibliometric perspective, introduced the concept and associated mechanisms of mitochondrial transfer, summarized the regulations of intercellular mitochondrial transfer on stem cell fate. Finally, the therapeutic application of mitochondrial transplantation in diseases and tissue regeneration has been reviewed, including recent clinical studies related to mitochondrial transplantation.

Mitochondrial transfer shows promise in modifying and reshaping the cellular properties of stem cells, making them more conducive to regeneration. Mesenchymal stem cells (MSCs)-derived mitochondria have shown multifaceted potential in promoting the revitalization and regeneration of cardiac, cutaneous, muscular, neuronal tissue. This review integrates novel research findings on mitochondrial transfer in stem cell biology and regenerative medicine, emphasizing the crucial translational value of mitochondrial transfer in regeneration. It serves to underscore the significant impact of mitochondrial transfer and provides a valuable reference for further exploration in this field.

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Keywords Mitochondrial transfer, Stem cell fate, Regenerative medicine, Mitochondrial therapeutics, Tissue repair

Background

Mitochondria, the semi-autonomous organelles that encapsulate mitochondrial DNA (mtDNA), are the powerhouses of the cell, with the production of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). They also play a crucial role in cellular fate regulation. Quiescent stem cells depend largely on glycolysis for energy consumption, while proliferation and differentiation of stem cells are tightly associated with mitochondrial biogenesis and the metabolic remodeling from glycolysis to OXPHOS to meet the increasing energy demand [1]. However, mitochondria are involved in various cellular processes beyond bioenergetics, including cellular signaling, immunomodulation, stress response, regulation of cellular homeostasis, differentiation, senescence, and apoptosis [2]. Mitochondria can be vertically inherited from parent to daughter cells during cell division. Recent studies have identified horizontal transfer of mitochondria, also known as intercellular mitochondrial transfer, in a variety of cells under both physiological and pathological conditions [3]. Among the various intercellular regulatory mechanisms over stem cell fate, the orchestration of mitochondrial transfer has been distinguished lately [4].

Mitochondrial transfer occurs between stem cells of autologous, allogenic or xenogeneic origin. For instance, rat bone marrow mesenchymal stem cells (BMMSCs) can internalize mitochondria from autogenous cells, leading to enhanced proliferation, migration, and osteogenic differentiation [5]. During coculture, human BMMSCs can receive mitochondria from adjacent allogeneic human vascular smooth muscle cells (VSMCs), T cells, or xenogeneic rat renal tubular cells, resulting in changes to their cellular status [6–8]. Functional mitochondria are preferentially donated to surrounding stem cells suffering stress from hypoxia, chemotherapy, irradiation, glucose deprivation, inflammation, etc., during coculture. Stem cells with extra mitochondria show improved ATP production, restored bioenergetics, alleviated oxidative stresses and enhanced cellular potentials [9]. Additionally, stem cells exert their pro-angiogenic and immunomodulatory effects by delivering mitochondria to target cells. For instance, BMMSCs show an immunosuppressive effect over T helper 17 (Th17) cells after delivering mitochondria to them during coculture, as indicated by a decrease in interleukin-17 production [10].

Based on the regulatory effects of mitochondrial transfer over stem cells, researchers have discovered the tremendous potential of mitochondrial therapeutics in disease treatment and tissue regeneration. Mesenchymal stem cells (MSCs) potentiated with exogenous mitochondria demonstrated superior tissue reparative effects compared to non-preconditioned counterparts when implanted into the injury sites of skin wounds and bone defects [11–14]. Moreover, mitochondria isolated from MSCs alone could achieve therapeutic effects in terms of pro-angiogenesis, immunomodulation, and regeneration when applied in diseases such as ischemia-reperfusion injury [15], osteoporosis [16], spinal cord injury (SCI) [17], muscle atrophy [18], and among others without the need for intact MSCs. Administration of autologous or allogeneic mitochondria into ischemic areas via epicardial or intracoronary injection was observed to improve postoperative cardiovascular function in patients with ischemia-reperfusion injury [19, 20]. The well-tolerated nature of mitochondrial transplantation by recipients validates its safety and feasibility as a novel therapeutic approach. Mitochondrial transplantation sheds light on a new possibility of stem cell-free regeneration, with reduced risks of immune responses and neoplasia.

However, the extent to which mitochondrial transplantation has advanced in the translation from preclinical studies to clinical trials remains unclear. The comprehensive regulation of mitochondrial transfer over stem cell fate and its huge potential in tissue regeneration have yet to be fully realized. The aim of this review is to provide a novel and comprehensive perspective on the translational research and application of mitochondrial transfer in regenerative medicine. We conducted a bibliometric analysis to predict the overall upsurging trend in mitochondrial transfer research in regenerative medicine based on literature from the past decade. Additionally, we explored the mechanisms and triggers for intercellular mitochondrial transfer, as well as the regulatory roles of mitochondrial transfer in stem cell fate. Furthermore, we delved into the therapeutic applications of mitochondrial transfer, especially focusing on recent advancements in clinical trials. Finally, we reviewed numerous emerging studies on mitochondrial transplantation for tissue regeneration and highlighted the challenges.

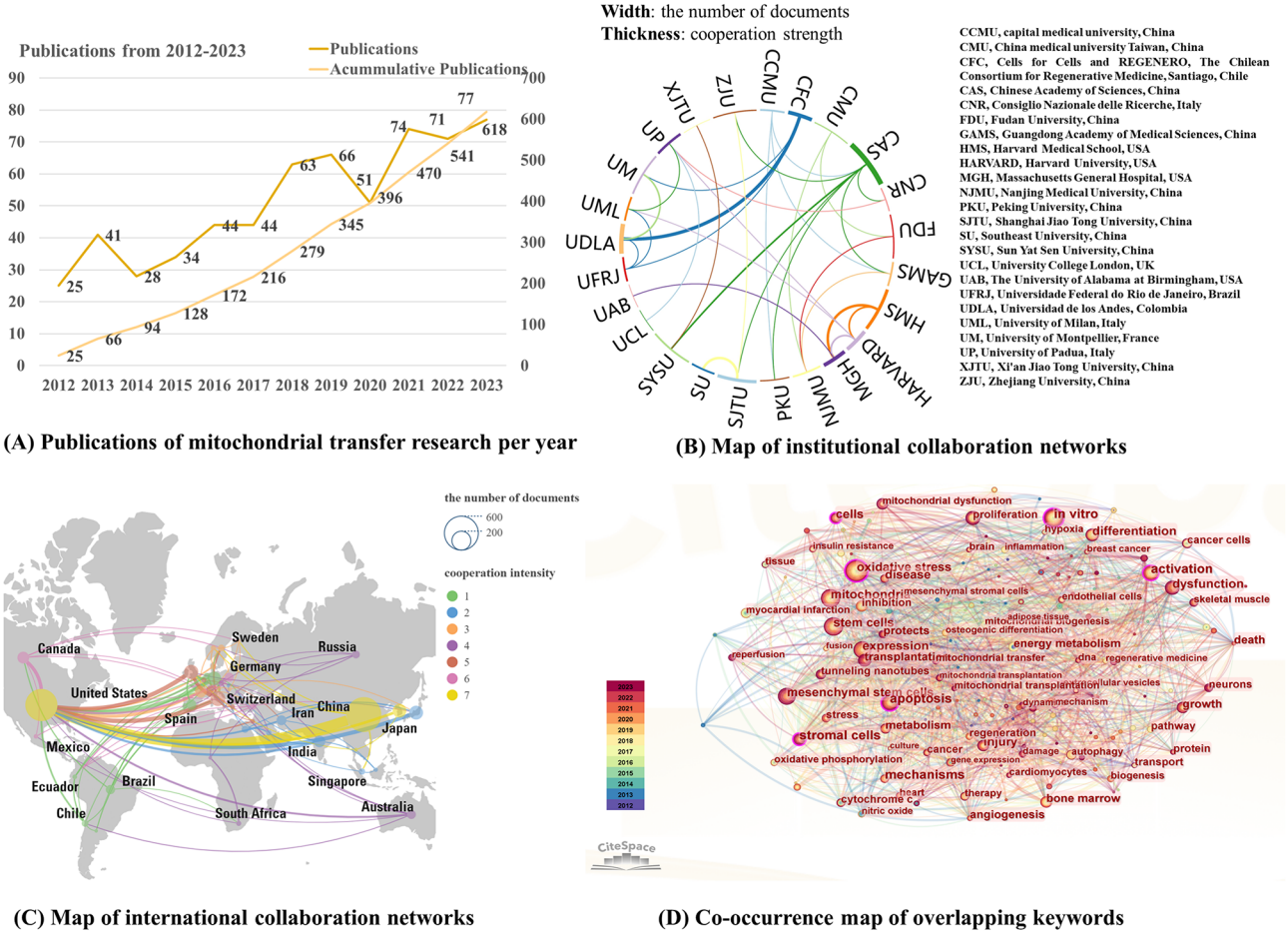
Global bibliometric mapping of mitochondrial transfer research for the period 2012–2023

Intercellular mitochondrial transfer has emerged as a novel intercellular regulatory mechanism over the past decade. It has been widely investigated for its role in regulating stem cell fate and promoting tissue regeneration. We performed a bibliometric analysis of mitochondrial transfer research in the field of stem cell biology and regenerative medicine by searching through the Web of Science Core Collection (WoSCC) database. The search

terms are as follows: TS= (mitochondria transfer OR mitochondria transplant* OR mitochondria donat* OR mitochondria isolat* OR free mitochondria OR mitochondria delivery) AND ((TS= (stem cell) AND TS= (cell fate OR differentiation OR proliferat* OR growth OR migrat* OR apoptosis OR cell death)) OR TS= (regenerative medicine OR regenerat* OR tissue engineering OR revital* OR revascul* OR angiogen* OR wound healing OR tissue repair)) AND DT= (Article OR Review) AND PY= (2012–2023) AND LA= (English). Literature information extracted from WoSCC was analyzed and visualized through CiteSpace, VOSviewer, and Scimago Graphica.

In recent years, there has been a significant increase in publications on mitochondrial transfer research within the context of stem cell regulation or tissue regeneration (Fig. 1A), with a notable surge since 2018. Prior to 2018, there was a burgeoning interest in mitochondrial transfer in the field of regeneration that began to gain attention. However, since 2018, there has been a notable surge in publication output in this field, signaling increased scholarly research and leading to more productive outcomes.

According to current curve fitting, it is expected that there will be a continued upward trend in this field. Scholars from different institutions across various countries in this research field have actively participated in identifying the characteristics, mechanisms, and potential clinical implications of mitochondrial transfer in stem cell-based regenerative medicine. Researchers from institutions such as Universidad de los Andes, Chinese Academy of Sciences, Massachusetts General Hospital, Harvard Medical School, Shanghai Jiao Tong University have taken the lead in both publication output and academic collaboration. The connecting line indicates that



(A) Publications of mitochondrial transfer research per year

(B) Map of institutional collaboration networks

(C) Map of international collaboration networks

(D) Co-occurrence map of overlapping keywords

Fig. 1 Global bibliometric analysis of mitochondrial transfer research from 2012 to 2023. (**A**) Since 2012, the number of publications in this research field has increased gradually. An upsurging academic attention from 2018 onwards can be indicated from this bar chart. (**B**) The map of the top 25 most productive institutions and their collaborations. The width of each column represents the number of published articles, while the thickness of the lines connecting two institutions represents the intensity of academic interaction between them. (**C**) The map of global collaboration networks between countries and/or regions. The size of nodes represents the quantity of published articles. The different colors and thickness of connecting lines indicate varying levels of cooperation between countries. (**D**) The co-occurrence map of overlapping keywords related to mitochondrial transfer and stem cell research. The frequency of occurrence is indicated by font size

two institutions have collaborated, with the thickness of the line representing the strength of the collaborative relationship (Fig. 1B). Scholars from the United States and China are among the most productive and collaborative in this field, as indicated by the size of nodes and the thickness of connecting lines, respectively (Fig. 1C). Moreover, a map of keywords co-occurrence analysis and cluster analysis are presented here as indicators for research hotspots (Fig. 1D). Keywords such as "mesenchymal stem cells", "stem cells", "mitochondria transfer/transplantation", "angiogenesis", "apoptosis", "proliferation", "tunneling nanotubes", "regeneration/regenerative medicine" and "oxidative phosphorylation" closely related to this topic have been frequently investigated over the past few years.

Mitochondrial transfer, as a novel intercellular regulatory mechanism, offers new possibilities for regeneration. It will continue to be a hot topic in stem cell biology and stem cell-based regeneration research. On-going in-depth research in regenerative medicine based on mitochondrial transfer is expected to lead the research front. Our bibliometric analysis indicates a significant relationship between stem cell fate, regeneration, and mitochondrial transfer. Emerging academic attention has been drawn in the field of mitochondrial transplantation in regenerative medicine.

Mechanisms and triggers for mitochondrial transfer

Early research has demonstrated the intercellular trafficking of mitochondria. When coculturing human endothelial progenitor cells (EPCs) and neonatal rat cardiomyocytes, transportation of MitoTracker-stained structures from cardiomyocytes to EPCs was observed [21]. This phenomenon of mitochondria delivery has been confirmed in various cells through many subsequent studies. While MSCs and progenitor cells were predominantly used as donors, induced pluripotent stem cells (iPSCs) were also utilized. The recipient cells include alveolar epithelium, cardiomyocytes, endothelial cells, macrophages, neural cells and MSCs. This kind of organelle exchange occurs in cells of different types and various species [22–26]. Islam et al. demonstrated that mitochondrial transfer could serve as a mechanism for stem cell-based therapy in vivo. Specifically, they administered BMMSCs to the airway of mice with acute lung injury. The BMMSCs were capable of releasing mitochondria, which were engulfed by damaged epithelial cells and subsequently reconstructed alveolar bioenergetics [27].

Mechanism of mitochondrial transfer

Based on the literature published so far, mitochondrial transfer has been achieved through three approaches: [1] mitochondria transport through transient cellular connections, such as tunneling nanotubes (TNTs) and connexin 43 (Cx43)-mediated gap junctional channels (GJCs) [2], mitochondria encapsulated in extracellular vesicles (EVs) and [3] free mitochondria released by donor cells for capture by target cells (Fig. 2).

Cellular connection-mediated mitochondrial transfer

The major cytoskeletal constituent of TNTs is F-actin. Components such as myosin Va and myosin X are recognized as critical mediators in target cell recognition and adhesion during TNT formation. Microtubules have also been detected in a few cell lines [32]. TNTs are capable of mediating intercellular communication over distances of 50–200 nm, facilitating transportation of organelles like endoplasmic reticulum, mitochondria, Golgi and endosomes in an actin-dependent manner under stress [33]. TNTs form intensively in response to bacterial invasion, viral infection, tumorigenesis, or tissue injury. Virus particles such as human immunodeficiency virus are also found to exploit TNTs as an infection highway to spread between cells [34]. TNTs also function in the tumor microenvironment and contribute to the survival, proliferation, and drug resistance of tumor cells by assisting the transfer of calcium flux, mitochondria, and cytokines. When confronted with reactive oxygen species (ROS)-inducing conditions, such as ultraviolet radiation, stroke, SCI, chemotherapy agents and trauma, compromised cells generate TNTs to transfer cellular components from adjacent healthy cells and survive the crisis [35, 36]. Healthy donor cells are also involved in the formation of TNTs, which help facilitate the transfer process [37]. However, the question of whether participation in TNT formation depends on the donor cell, recipient cell, or both remains unsolved.

When neurons and astrocytes are under stress, p53 activation triggers the subsequent upregulation of epidermal growth factor receptor (EGFR) expression and stimulation of the Akt/phosphoinositide 3-kinase (PI3K)/mTOR signaling cascade. This p53-mediated regulation via EGFR or the Akt/PI3K/mTOR pathway further promotes excessive production of M-Sec (also known as tumor necrosis factor α-induced protein 2). In concert with the RalA small GTPase and the exocyst complex, M-Sec facilitates F-actin polymerization, ultimately driving the formation of TNTs from the plasma membrane [33]. Once TNTs are formed, the outer membrane proteins of mitochondria, such as Miro1 and Miro2, serve as motor proteins and combine with accessory proteins like kinesin to facilitate cargo transfer [38]. Knockdown of Miro1 expression in exogenously administered MSCs impaired the rescue of inflammation in a murine model of rotenone-induced airway epithelial injury, likely due to inhibition of mitochondrial transfer [38]. Overexpression of Miro1 in donor MSCs increased mitochondrial

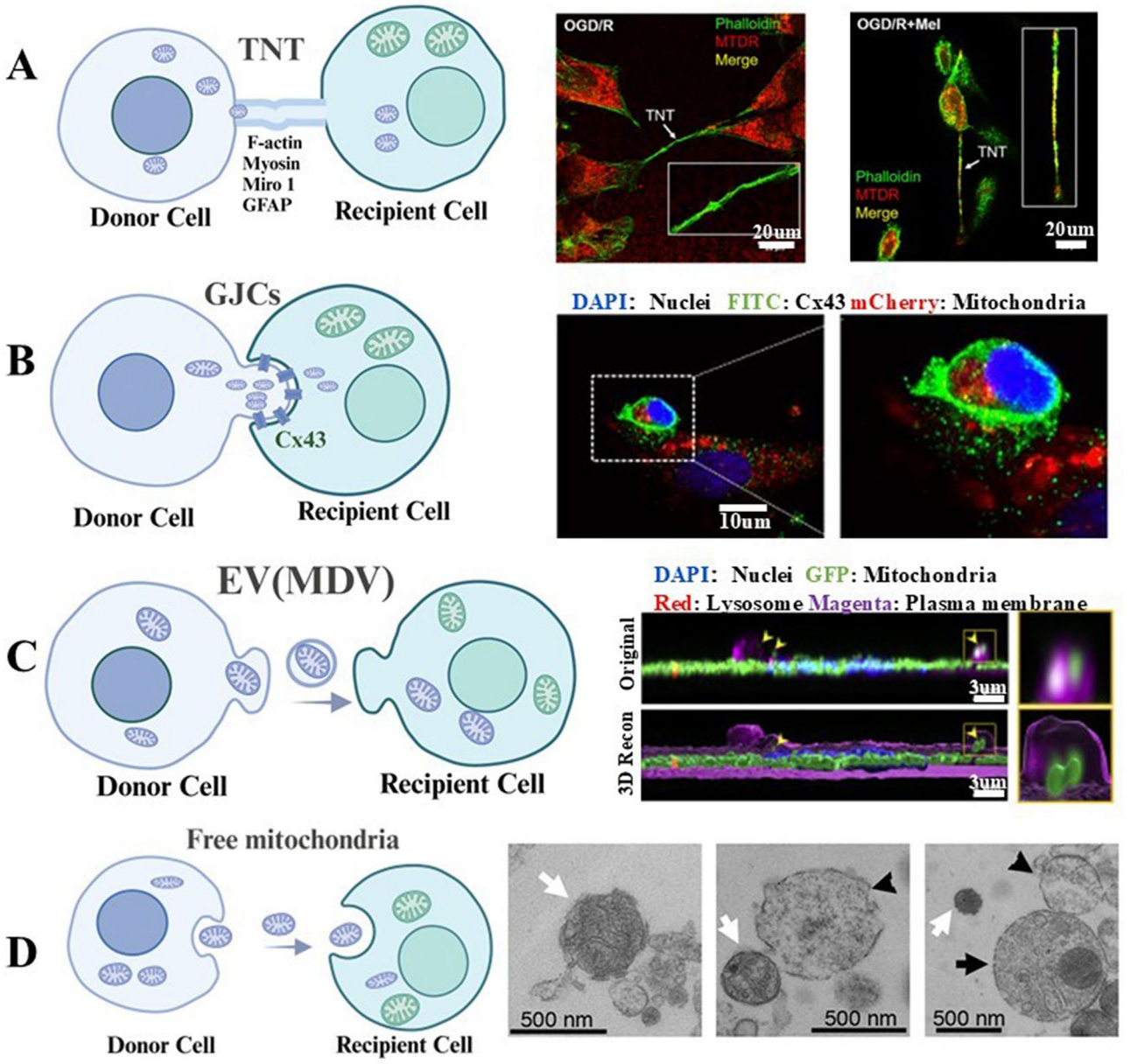


Fig. 2 Schematic diagram and illustrations for the mechanisms of mitochondrial transfer. (**A**) Mitochondria transport through tunneling nanotubes (TNTs). F-actin, myosin, Miro1 and glial fibrillary acid protein (GFAP) can function as regulators of this transfer process. An illustration of mitochondria (MTDR)-laden TNTs (phalloidin) formed by murine hippocampal HT22 cells exposed to oxygen glucose deprivation/reoxygenation (OGD/R) and rescued by melatonin (Mel). Reproduced under the terms of CC-BY 4.0 license [28]. Copyright 2021 The Authors. Published by John Wiley & Sons. (**B**) Mitochondrial transfer via connexin 43 (Cx43)-mediated gap junctional channels (GJCs). Cx43(FITC) regulate mitochondrial (mCherry) movement from bone marrow mesenchymal stem cells (BMMSCs) to hematopoietic stem cells (HSCs) stimulated by hydrogen peroxide (H_2O_2) treatment. The nuclei are labeled with DAPI. Reproduced under terms of the CC BY-NC-ND 4.0 license [29]. Copyright 2019, The Authors, published by PNAS. (**C**) Mitochondria encapsulated in extracellular vesicles (EVs) or mitochondria-derived vesicles (MDVs). During osteogenic differentiation of osteoblasts, mitochondria(arrowheads) or MDVs (arrowheads) about to be secreted, plasma membrane (Magenta)-enclosed mitochondria (GFP) or MDVs secreted are indicated in confocal images. The nuclei are labeled with DAPI. Reproduced under the terms of CC-BY 4.0 license [30]. Copyright 2023 The Authors. Published by Elsevier Inc. (**D**) Free mitochondria excreted by donor cells and will be captured by target cells in a heparan sulfate-dependent way. TEM visualization of mitochondria (white arrows), mitochondria-containing microparticles (black arrows), and microparticles (black arrowheads) released from thrombin-activated platelets, verified by three-dimensional confocal image reconstruction of the supernatant of thrombin-activated platelets. Reproduced under the terms of CC-BY-NC-SA license [31]. Copyright 2014 The Authors. Published by Elsevier Inc. Schematic diagram part is created with Biorender

transfer in recipient cells [37, 39]. Furthermore, during oxidative stress and apoptosis, glial fibrillary acid protein (GFAP), an intermediate filament cytoskeleton protein, was highly expressed within TNT structures of glioblastoma cell lines, suggesting that GFAP might serve as another structural component of TNTs [40]. Wang et al. found that cell adhesion regulated by intercellular adhesion molecule-1 (ICAM-1) was crucial for TNT-mediated mitochondrial transfer. Treatment with neutralizing antibodies against ICAM-1 led to a decrease in mitochondrial transfer [7].

GJCs, which consist of connexin subunits, are formed by two adjacent cells through docking their hemichannels, respectively. The protective role of exogenously administered BMMSCs in mitigating organ injury or inflammation had been demonstrated to occur through Cx43-dependent mitochondrial transfer. When BMMSCs approached the alveoli, Cx43-based GJCs were formed by the donor cells, through which healthy mitochondria were delivered from BMMSCs to the impaired alveolar epithelium [27]. When BMMSCs suffered from irradiation damage, the transplantation of hematopoietic stem and progenitor cells (HSPCs) also showed similar rescue effect in a Cx43-dependent manner [41]. A study by Yao et al. found that Cx43-mediated mitochondrial transfer occurred through the regulation of TNT formation in human iPSC-derived MSCs [22]. Meanwhile, there were occasions where mitochondrial transfer was not influenced by inhibition of Cx43 expression, indicating that the complexity of cellular connections in coordinating mitochondrial transfer was not fully understood [42]. When osteocytes were under oxidative stress, Cx43 was found to have functions beyond transfer channels as it translocated into the mitochondrial inner membrane, directly interacted with ATP synthase subunit ATP5J2, and regulated mitochondrial homeostasis [43].

EV-mediated mitochondrial transfer

As a pivotal mediator of intercellular trafficking, EVs not only carry cellular components such as microRNA, mRNA, cytokines, and functional enzymes, but are also in charge of delivering intact organelles like mitochondria. This approach of organelle transfer makes mitochondria release into the bloodstream and intercellular microenvironments possible. EV-encapsulated mitochondria in circulation originated from platelets, endothelial cells, and leukocytes [44, 45] and they expressed tetraspanin markers such as CD9, CD63 and CD81. EV-mediated mitochondrial transfer is closely related to multifaceted cell fate regulation. Variations in the size and characteristics of EVs may be associated with diverse roles.

One way to regulate cell fate is through mitochondrial quality control, which eliminates damaged mitochondria to preserve tissue function and stability. Usually the accumulation of dysfunctional mitochondria in cells are prevented via mitophagy, mediated by PTEN-induced kinase 1 (PINK1)/Parkin pathway. In detail, damaged mitochondria with the loss of mitochondrial membrane potential (MMP) ensure the accessibility of PINK1. PINK1 binds to the translocase of the outer membrane (TOM) and recruits a cytoplasmic E3-ubiquitin ligase, Parkin, leading to a spatial conformational change of Parkin. Parkin ubiquitinates mitochondrial proteins as a label for degradation and together with the lysosome, constitutes autophagosome [46]. When this autophagy pathway was inactivated due to age or genetic mutations, such as *Rab7* knockdown (Rab7 is a GTPase in charge of lysosome formation) in cardiomyocytes, there was a distinct mechanism of mitochondrial exclusion through increased secretion in larger EVs (300–600 nm in size) [47]. Cardiomyocytes have also been reported to release large EVs (3.5–4 μm) containing damaged mitochondria, known as exophers. These EVs or exophers are then captured and degraded by macrophages residing in the myocardium [48]. In order to maintain thermogenesis in response to thermogenic stimuli, adipose tissues secrete damaged mitochondria containing EVs and export them for elimination by macrophages. In brown adipocytes, the ejection of oxidized, damaged mitochondria due to thermogenic stimuli occurs through the formation of approximately 100-nm-diameter mitochondria-derived vesicles (MDVs). The exportation of MDVs, just like mitophagy, was also regulated by PINK1 and Parkin pathway [49, 50]. Since different mitochondrial trajectories are involved, the downstream pathways of PINK1/Parkin determining mitophagy or MDV ejection, or mitochondrial transfer are disparate and largely unknown. EVs-associated mitochondrial transfer, as an alternative pathway of cellular quality control, are indispensable for the clearance of damaged mitochondria and the maintenance of normal cellular physiological activity.

EV-encapsulated mitochondria can act in a way to support the functions of recipient cells. In mature osteoblasts, mitochondria donuts and MDVs are formed and excreted to boost the differentiation and maturation of osteoprogenitors, which function in a positive feedback mechanism to accelerate bone regeneration [30]. EV-associated mitochondria produced by neural stem cells (NSCs) were able to integrate into the mitochondrial network of mononuclear phagocytes by endocytosis, leading to inhibition of the metabolic switch toward pro-inflammation. Moreover, functional mitochondria containing EVs derived from NSCs could also enter mtDNA-depleted cells to restore their mitochondrial function [51].

Based on the above discussion, mitochondria found in EVs can either be intact, potent, or dysfunctional. This

implies that mitochondria exported by cells in different states vary in their conditions, leading to distinct impacts on target cells. Future studies are needed to elucidate the mechanisms by which mitochondria-containing EVs dock onto target cells, enter the cytoplasm of specific cells, escape cellular degradation, and exert their effects once they are captured by target cells.

Freely released mitochondria

This form of extracellular mitochondria was initially identified in the bloodstream and in the culture media of certain human colon cancer cell lines. Further research has shown that these cell-free mitochondria originated from both healthy and tumor cells in different species and have a diameter of about 0.5–1 μm [52]. Circulating mitochondria originate from activated platelets, adipocytes and possibly other cell sources and they express the outer mitochondrial membrane protein TOM22. However, they are negative for extracellular vesicle markers such as CD9, CD63 and CD81 [31, 53], which is evidenced by the absence of EV membrane structures under transmission electron microscope (TEM) observation. The maintenance of transmembrane potential and integrity of the mitochondrial genome indicate they are intact in structure.

When mitochondria are extracted from human plasma, oxygen consumption cannot be detected in plasma, suggesting circulating free mitochondria are respiration-competent [31]. However, one study suggested that the electron transport chain (ETC) of free mitochondria in circulation was nonfunctional, so they are incapable of OXPHOS [54]. Likewise, cerebrospinal fluid samples from rat models and patients suffering from subarachnoid hemorrhage have shown increased levels of collapsed mitochondria. Those with lower MMP showed greater disease severity. Recovery of MMP on the third-day post-injury was significantly associated with good outcomes at 3 months [55].

Free mitochondria are captured by recipient cells through endocytosis, an active transport process for engulfing extracellular material. In different scenarios based on the cell types, the underlying molecular machinery for mitochondrial internalization through endocytosis can vary. Dynamin-dependent clathrin-mediated endocytosis is the major route for the most studied mitochondrial transfer. The internalization of platelet mitochondria, either free or EV-encapsulated, is completely suppressed in adipose-derived mesenchymal stem cells (ADSCs) by dynasore, an inhibitor of dynamin-dependent clathrin-mediated endocytosis [13]. Moreover, mitochondria isolated from cardiomyocytes are internalized by MSCs through this pathway as well [56].

When mitochondria isolated from human astrocytes are co-incubated with human glioma cells undergoing glucose deprivation, exogenous mitochondria are internalized via endocytosis to rescue aerobic respiration. The slowed glycolysis decreases NAD^+ /NADH (oxidized/reduced form of nicotinamide-adenine dinucleotid) transformation in glioma cells. CD38, a member of the NAD^+ glycohydrolase family, catalyzes the cyclization of elevated extracellular NAD^+ to intracellular cyclic ADP-ribose (cADPR), leading to the release of Ca^{2+} to promote cytoskeleton remodeling and plasma membrane invagination. Thus, mitochondrial endocytosis in this context is mediated by activation of NAD^+-CD38-cADPR-Ca^{2+} transduction pathways [57] (Fig. 3).

Macropinocytosis is another form of endocytosis, which does not depend upon dynamin or require the particle to interact with receptors on plasma membranes. When transplanting mitochondria derived from human uterine endometrial gland-derived mesenchymal cells into cardiomyoblasts, mitochondrial transfer can be undermined due to inhibition of Na^+/H^+ exchange with ethyl isopropyl amiloride, suggesting the involvement of micropinocytosis in mitochondrial internalization [58]. A similar internalization route happens when the transfer of isolated mitochondria into osteosarcoma cells [59]. Genetic, enzymatic, and pharmacological disruption of heparan sulfate biosynthesis led to a decrease in mitochondrial uptake by recipient cells, validating heparan sulfate as an indispensable molecule for mitochondria internalization [60].

Currently, the involvement of disparate endocytic pathways is mainly verified by cellular morphological changes and the application of pharmacological inhibitors. Based on evidences acquired so far, it remains challenging to fully decipher the mechanisms by which mitochondria are taken up by recipient cells. The reasons for the differences in endocytosis pathways among different species, cell types, and modes of mitochondrial delivery are not fully elucidated, making it difficult to classify the various endocytosis processes involve in this complex phenomenon.

Fate of mitochondria after internalization

It has been supported by multiple studies that metabolic improvement, aerobic respiration and rescuing effects can occur due to mitochondrial transfer [13, 61, 62]. However, after entering a recipient cell via any approach, the fate of mitochondria and how they participate in cellular activity are largely unknown. One assumption suggested that mitochondria could escape from the endosomal compartment and integrate with the endogenous mitochondrial network directly [63]. Another putative intracellular trajectory of exogenous mitochondria was to be entrapped in endosomes, respired within endosomes, and transported materials into the cytoplasm [64]. Recently, researchers found that exogenous

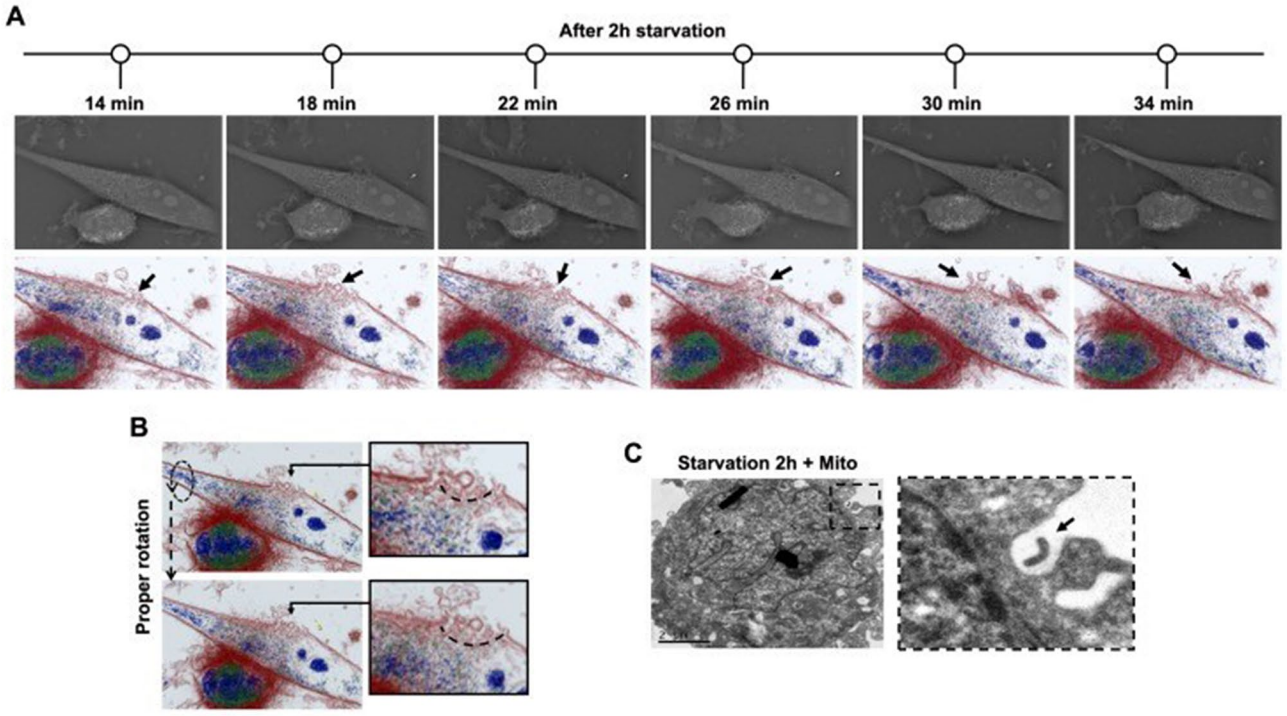


Fig. 3 The ongoing process of human glioma cells engulfing free mitochondria via endocytosis. (**A**) The dynamic process of endocytosis during starvation treatment was captured by 3D tomographic microscope colored by refractive index, not staining. (**B**) Endocytosis observed after proper rotation of the stereoscopic structure (**C**) TEM revealed that invagination of the plasma membrane encapsulated isolated mitochondria. Reproduced under the terms of CC BY-NC 4.0 license [57]. Copyright 2019. The Authors. Published by Ivyspring International Publisher

mitochondria originated from human umbilical cord-derived mesenchymal stem cells (hUCMSCs) and ADSCs entered healthy cells either through intercellular connections or endocytosis, acted as a trigger for mitophagy [56, 65]. It is the priming effect of donor mitochondria degradation on mitochondrial biogenesis that matters in the improvement of cellular capacity. Exogeneous mitochondria, regardless of whether they are intact, depolarized or mtDNA-free, do not need to be functionally incorporated into recipient cells, as they are all capable of activating mitophagy, enhancing mitochondrial biogenesis and improving respiration and cellular functions [65]. With the speculation that cells preferentially use endogenous mitochondria for nutrient metabolism and utilize exogenous mitochondria in response to a metabolic emergency [66], it is reasonable to assume that the fate of exogenous mitochondria differs when internalized by damaged cells with or without inherent deficiency in endogenous mitochondrial function.

There are still several details that require investigation, including the mechanisms by which mitochondria enter cells, the trajectory of exogenous mitochondria, how they integrate into the recipient cell's mitochondrial networking, and their role in remodeling the cellular status after absorption. A deep understanding of these processes will facilitate therapeutic applications of mitochondrial transfer.

The triggers for mitochondrial transfer

Mitochondrial transfer happens naturally in a physiological context, even without additional stimuli; the coculture of two cell types will lead to mitochondrial transfer and in some cases, bidirectional mitochondria exchange [6, 8, 67]. However, mitochondrial transfer has mostly been observed in disease models associated with cell damage (Fig. 3). Particularly, the accumulation of cellular ROS caused by the majority of stimuli, such as ischemia-hypoxia, radiation exposure, and mtDNA deletion, which drives the mitochondrial transfer process, and facilitates mitochondrial transport in a more efficient manner compared to normal conditions [39, 68, 69]. The PI3K/AKT pathway is a frequently studied signaling pathway that mediates mitochondrial transfer. The activation of PI3K/AKT pathway due to ROS accumulation can result from very different stimuli, and the downstream effector molecules across different cell types and studies. For instance, both inflammation and nanomaterials are capable of inducing PI3K activation and AKT phosphorylation, the former leads to Cx43 channel opening and the latter gives rise to the formation of TNTs [29, 70]. This indicates that our understanding of the triggering mechanisms and

principles of mitochondrial transfer is still far from sufficient. The major triggers for mitochondrial transfer will be discussed as follows.

Inflammation

Bacterial infections such as pyocyanin, lipopolysaccharide (LPS), and *S. typhimurium* could elevate ROS levels in stem cells [71]. In the bone marrow microenvironment, bacterial pathogens could stimulate mitochondrial transfer from BMMSCs to hematopoietic stem cells (HSCs), which modified the bioenergetic status of HSCs from glycolytic metabolism toward OXPHOS to accommodate emergency granulopoiesis. The trigger of mitochondrial transfer was dependent on ROS-induced oxidative stress, which activated PI3K, mediated AKT kinase phosphorylation and opened Cx43 channels [29] (Fig. 4A).

Culture condition

Mitochondrial transfer supports recipient cells with mitochondrial dysfunction in overcoming energy crises and improving their metabolic status [72, 73]. The formation of TNTs is controlled by certain in vitro culture conditions (Fig. 4B). The onset of ischemia or hypoxia initiated an overexpression of TNTs or accelerated release of mitochondria-loaded EVs. Oxygen-glucose deprivation (OGD) is a common approach used to induce excess ROS production, mimicking the tissue microenvironment of ischemia-reperfusion injury. TNT formation was observed at a higher frequency between the BMMSCs and the injured human umbilical vein endothelial cells (HUVECs) cultivated under OGD than in cocultivation under normal conditions [74]. BMMSCs conveyed mitochondria more efficiently when recipient cells were exposed to OGD [17, 39]. In addition, hyperosmotic

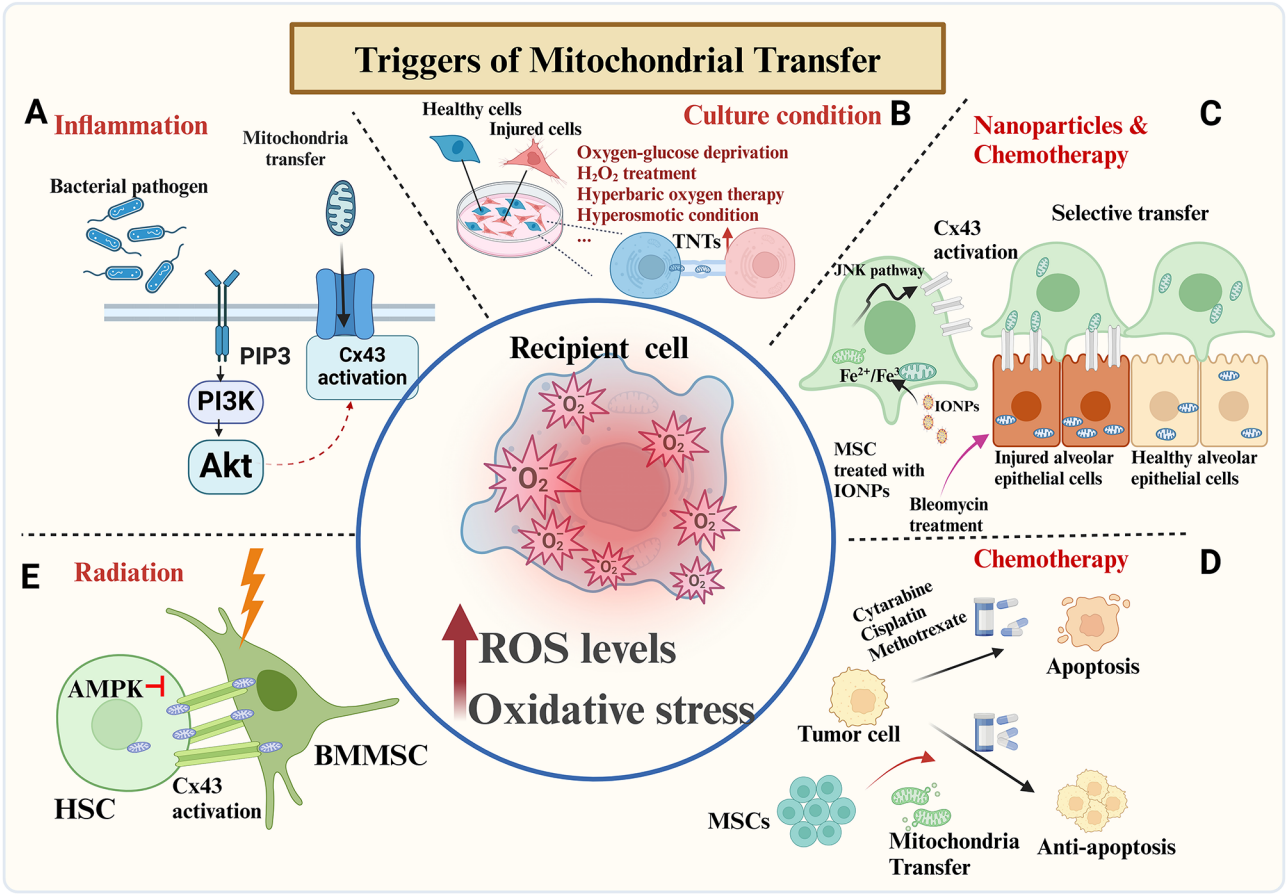


Fig. 4 Triggers of Mitochondrial Transfer. In most cases, the recipient cells are provoked by diverse stimuli and become vulnerable with elevated reactive oxygen species (ROS) levels and intense oxidative stress. (**A**) Bacterial pathogen induces cellular oxidative stress, then activates phosphoinositide 3-kinase (PI3K), leads to AKT phosphorylation and opening of Cx43 channels, and eventually gives rise to mitochondrial transfer. (**B**) Recipient cells going through oxygen-glucose deprivation (OGD), H_2O_2 treatment, hyperbaric oxygen therapy, or hyperosmotic conditions will promote the efficiency of mitochondrial transfer. (**C**) Iron oxide nanoparticles (IONPs) treatment promotes Cx43 expression in MSCs and increases mitochondria donation, contributing to the rescue of bleomycin-treated alveolar epithelial cells. Mitochondrial transfer selectively happens in injured epithelial cells instead of healthy cells. (**D**) More mitochondrial transfer can be observed when recipient cells go through chemotherapy stress such as tarabine, cisplatin, methotrexate (MTX) treatment, etc. (**E**) When the bone marrow microenvironment suffers from radiation, transplanted exogenous healthy hematopoietic stem cells (HSCs) will donate their mitochondria to injured bone marrow mesenchymal stem cells (BMMSCs) via Cx43 channels. Schematic diagram created with Biorender

conditions have been shown to drive the mitochondrial transfer process. The efficiency of mitochondrial transfer between human corneal epithelial cells was slightly elevated when exposed to a hyperosmotic culture medium [75]. Approaches such as superoxide administration and preconditioning cells through hyperbaric oxygen therapy have been shown to enable mitochondria to migrate towards susceptible cells [29, 76].

Chemotherapy stress

More TNT expression and more mitochondrial transfer had been detected between HUVECs and BMMSCs when HUVECs were pretreated with cytarabine (Ara-C). The donation of mitochondria from BMMSCs has been shown to help recipient endothelial cells combat chemotherapy stress by alleviating apoptosis, improving proliferation, enhancing transmembrane migration capacity and increasing capillary angiogenic potential [77]. Murine MSCs are also capable of donating healthy mitochondria to rescue murine NSCs from cell loss caused by cisplatin treatment [37]. Tumor cells have also exploited this rescue effect of mitochondrial transfer (Fig. 4D). Wang et al. found that T cell acute lymphoblastic leukemia (T-ALL) that were exposed to chemotherapeutic drugs transferred a significantly higher amount of mitochondria to BMMSCs than they received from BMMSCs, even when both were exposed to antineoplastic drugs equivalently. This transfer helped T-ALL survive chemotherapy stress by reducing intracellular ROS levels [7].

Irradiation

Golan et al. found that when the bone marrow microenvironment suffered from irradiation preconditioning, transplanted healthy HSCs were capable of recovering damaged BMMSCs by donating their functional mitochondria to BMMSCs. The onset of mitochondrial transfer was due to an elevated intracellular ATP concentration in hematopoietic cells, which activated the purinergic receptor P2RX7 and inhibited adenosine 5′-monophosphate–activated protein kinase (AMPK) activation in HSCs. This led to the stimulation of Cx43-dependent mitochondrial transfer to BMMSCs [41] (Fig. 3E). Another group has found that irradiation could affect the formation of TNTs in glioblastoma stem cells, which further supported the idea that radiotherapy might have an impact on mitochondrial transfer [78].

Other triggers

The process of mitochondrial transfer can occur through different approaches, and thus, disparate stimuli may activate diverse pathways to facilitate the exchange of organelles. One research team applied iron oxide nanoparticles (IONPs) to promote Cx43 expression in human placental MSCs and increased mitochondria donation, which contributed to the rescue of bleomycin-treated murine alveolar epithelial cells. The injured cells showed a remarkable increase in MMP and intracellular ATP levels while experiencing a decrease in ROS levels [79] (Fig. 4C). According to Lin et al., nanomaterials such as cobalt nanoparticles, titanium dioxide nanoparticles, and multi-walled carbon nanotubes are able to induce oxidative stress in U251 human glioma cells, leading to the activation of PI3K/AKT/mTOR pathway, eventually initiating the formation of TNTs and mitochondrial transfer [70]. Another group found melatonin could promote intercellular mitochondrial transfer via TNTs after ischemic-like injury [28]. These findings offer insightful views on how known mechanisms of mitochondrial transfer can be exploited to intentionally intensify mitochondria delivery for disease treatment.

Cellular effects of mitochondrial transfer on stem cells

Mitochondrial transfer exerts multi-dimensional and multi-level regulation on stem cell fate (Table 1). First, this phenomenon occurs between cell types of autologous, allogeneic, or xenogeneic origins [6, 37, 68]. Second, MSCs serve as both critical donor cells and indispensable mitochondrial recipients in this research field. As donor cells, MSCs can rescue stressed stem cells by delivering healthy mitochondria, decreasing oxidative stress, supporting metabolic homeostasis, and reducing apoptosis [29, 68]. Healthy stem cells exhibit enhanced proliferation, migration and viability following the internalization of MSC-derived mitochondria [5, 14]. MSCs can also absorb mitochondria from other somatic cells or stem cells, resulting in a variety of cellular effects [5, 68, 80]. Finally, mitochondria exhibit functional differences depending on the cellular conditions of donor cells. For instance, mitochondria isolated from cells pretreated with hydrogen peroxide (H_2O_2) or chemotherapeutic agents such as Ara-C and MTX induce opposing cellular effects in stem cells, including elevated intracellular ROS levels and activation of autophagy [7, 81]. Conversely, when donor cells are preconditioned with agents like N-acetyl-L-cysteine (NAC) and L-ascorbic acid 2-phosphate (AAP), the resulting "energized" mitochondria demonstrate protective capabilities by reducing oxidative stress and stabilizing the MMP in H_2O_2-treated ADSCs [68].

Several representative cellular effects of mitochondrial transfer on stem cell fate are illustrated in Fig. 5.

Proliferation

Stem cells convert from a quiescent to a proliferative state, accompanied by a metabolic shift toward OXPHOS, leading to ROS accumulation and adversely

Table 1 Mitochondrial transfer regulates stem cell fate

Origin of mitochondria	Donor cells	Recipient cells	Transferred cargoes	Route	Cellular effects on recipient cells	Ref.
Autologous	Rat BMMSCs	Rat BMMSCs	Healthy mitochondria	Mitochondrial isolation and internalization (low temperature and centrifugation)	Enhanced proliferation, migration, increased osteogenesis upon osteogenic induction and promoted bone defect healing potential in vivo	[5]
	Human PDLSCs cultured on high stiffness hydrogel	Human PDLSCs cultured on low stiffness hydrogel	Healthy mitochondria	Mitochondrial isolation and internalization (low temperature and centrifugation)	Reversed inhibitory effects of low stiffness hydrogel on osteogenic differentiation	[80]
	Human ADSCs (Combined treatment of NAC and AAP)	H_2O_2-treated human ADSCs	"Energized" mitochondria due to NAC and AAP pretreatment	Cell coculture (TNTs)	Decreased oxidative stress, stabilized MMP of H_2O_2-treated ADSCs, enhanced mitophagy and eliminated damaged mitochondria	[68]
Allogenic	Mice MSCs	Cisplatin treated Mice NSCs	Healthy mitochondria	Cell coculture (TNTs)	Reduced cell death	[37]
	Human ADSCs from younger patients	Human ADSCs from elderly patients	Healthy mitochondria	Mitochondrial isolation and internalization (coincubation)	Enhanced proliferation, migration, and differentiation in vitro; improved skin repair in vivo	[14]
	Human Platelets	Human ADSCs	Healthy mitochondria	Cell coculture (Dynamin-dependent clathrin-mediated endocytosis)	Stimulated pro-angiogenic potential and wound-healing efficacy	[13]
	Mice BMMSCs	Mice HSCs exposed to LPS or *S. typhimurium*	Healthy mitochondria	Cell coculture (Cx 43 Gap Junctions)	Increased mitochondrial mass, converted metabolic status from glycolysis toward OXPHOS	[29]
	Human lymphocytes	Schizophrenia-derived iPSCs	Healthy mitochondria	Mitochondrial isolation and internalization (coincubation)	Improved differentiation into neurons	[82]
	Mice HSCs	Mice BMMSCs after irradiation	Healthy mitochondria	Cell coculture (Cx 43 Gap Junctions)	Boosted bone marrow stroma regeneration and hematopoietic reconstruction after irradiation; increased colony formation and reduced apoptosis	[41]
	Mice liver	HSPCs of Polg mice (a model of mitochondrial dysfunction)	Healthy mitochondria	Mitochondrial isolation and internalization (centrifugation and coincubation)	Improved hematopoietic engraftment and lymphoid output; promoted multilineage hematopoietic potential	[83]
	Human placenta or peripheral blood	HSPCs from patient with mtDNA disorder	Healthy mitochondria	Mitochondrial isolation and internalization (centrifugation and coincubation)	Maintained HSPC viability or ability to form colonies in vitro, improved oxygen consumption; enabled higher long-term hematopoietic engraftment in vivo when intravenously injected HSPCs in mice	[83]
	Human VSMCs	Human BMMSCs	Healthy mitochondria	Cell coculture (TNTs)	Promoted BMMSCs proliferation, did not induce BMMSCs differentiation toward VSMCs	[8]
	Ara-C, MTX-treated human T cell acute lymphoblastic leukemia	Human BMMSCs	Damaged mitochondria	Cell coculture (TNTs)	Increased intracellular ROS levels in BMMSCs	[7]
	Human SHED	Human ESCs	Healthy mitochondria	Cell coculture (TNTs)	SHED were used as a substratum for ESCs culture to induce neural differentiation	[84]

Table 1 (continued)

Origin of mitochondria	Donor cells	Recipient cells	Transferred cargoes	Route	Cellular effects on recipient cells	Ref.
	H_2O_2-treated human cardiomyocytes or HUVECs	Human ADSCs	Damaged mitochondria	Cell coculture (TNTs)	Activated autophagy, induced HO-1 expression and stimulated mitochondrial biogenesis	[81]
Xenogeneic	Rat renal tubular cells	Human BMMSCs	Healthy mitochondria	Cell coculture (TNTs)	Expressed renal-specific Tamm-Horsfall protein and differentiated into kidney tubular cells	[6]

Abbreviations: BMMSCs, bone marrow mesenchymal stem cells; PDLSCs, periodontal ligament stem cells; ADSCs, adipose-derived stem cells; H_2O_2, hydrogen peroxide; NAC, N-acetyl-L-cysteine; AAP, L-ascorbic acid 2-phosphate; TNTs, tunneling nanotubes; MMP, mitochondrial membrane potential; MSCs, mesenchymal stem cells; NSCs, neural stem cells; HSCs, hematopoietic stem cells; LPS, lipopolysaccharide; Cx34, Connexin 43; OXPHOS, oxidative phosphorylation; iPSCs, induced pluripotent stem cells; HSPCs, hematopoietic stem and progenitor cells; mtDNA, mitochondrial DNA; VSMCs, vascular smooth muscle cells; Ara-C, cytarabine; MTX, methotrexate; ROS, reactive oxygen species; SHED, stem cells from human exfoliated deciduous teeth; ESCs, embryonic stem cells; HUVECs, human umbilical vein endothelial cells; HO-1, heme oxygenase-1

restricting cell growth [85]. When stem cells receive extra mitochondria, which function as central modulators of redox homeostasis, they show decreased ROS levels and increased cell viability [7, 45]. When coculture of human BMMSCs with VSMCs from human coronary artery, BMMSCs and VSMCs were capable of exchanging mitochondria. The consequence of receiving mitochondria from VSMCs was an upregulation in BMMSC cell growth [8]. Autologous mitochondria transplantation of murine BMMSCs has been shown to increase the proliferative potential of recipient cells, as indicated by Ki67 staining and higher expression of related marker, v-myc avian myelocytomatosis viral oncogene homolog (C-MYC). The proliferative potential and postponement in senescence were maintained even after 9 passages, according to CCK8 results and β-galactosidase staining, compared to BMMSCs that were not treated [5]. BMMSCs that received HSPCs-derived mitochondria showed improved cell growth and proliferation in vivo. When HSPCs were transplanted in the bone marrow microenvironment of irradiated mice, BMMSCs that had received mitochondria were able to proliferate more than those that did not receive mitochondria, as evidenced by increased colony formation and reduced apoptosis [41]. When BMMSCs received damaged mitochondria from stressed HUVECs, the engulfed mitochondria were degraded through mitophagy, which upregulated the expression of the cytoprotective heme oxygenase-1 (HO-1) enzyme. Through triggering mitochondrial biogenesis, HO-1 stimulated an anti-apoptotic response in BMMSCs [81].

Migration

Cancer cells are capable of capturing mitochondria from heterogeneous cancer cells or normal stem cells to facilitate their invasion [86, 87]. This increase in migration due to mitochondria absorption also works for normal cells. When incorporating platelets-derived mitochondria into human dermal fibroblasts, the latter exhibited better scratch wound healing potential with improvement in both migratory and proliferative potential [88]. BMMSCs absorbed autologous mitochondria showed enhanced migration ability according to the result of scratch wound healing assay, vertical migration test and cell tracking [12]. When BMMSCs donated mitochondria to HUVECs, the HUVECs showed enhanced transmembrane migration potential and elevated capillary formation on Matrigel [77]. The promotion of migration by mitochondrial transfer is particularly significant for wound healing and tissue regeneration.

Pro-angiogenesis

Mitochondria transplantation has been identified to promote skin wound healing, and facilitate recovery from ischemia/reperfusion-induced injury of cardiac or

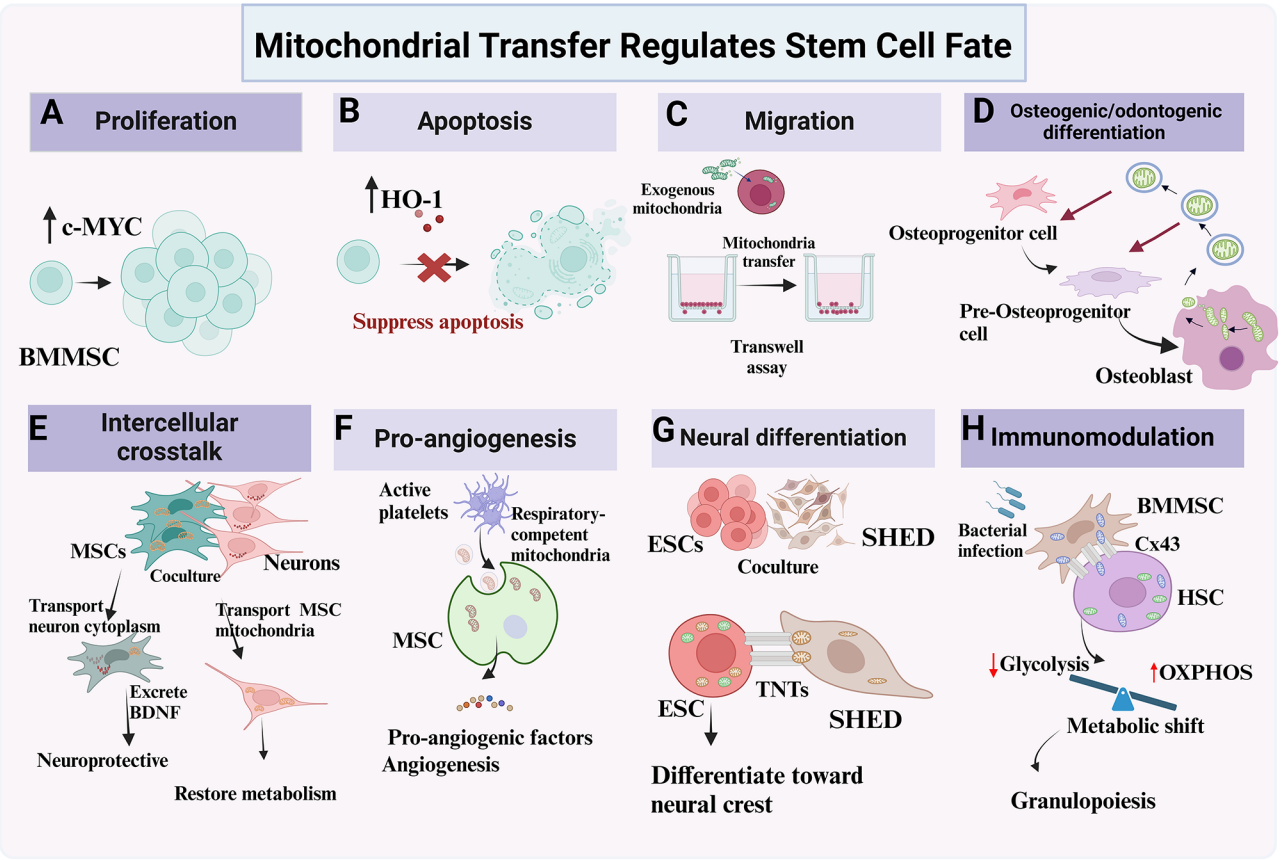


Fig. 5 Mitochondrial Transfer Regulates Stem Cell Fate. (**A**) Stem cells show enhanced proliferation after mitochondrial transfer, indicated by upregulated c-MYC expression. (**B**) The apoptosis of stem cells is inhibited with upregulated HO-1 expression after mitochondrial transfer. (**C**) Stem cells modified by mitochondrial transfer show improved migration potential. (**D**) Mitochondrial fission and donut formation increase during osteoblast maturation. Osteoblast secretes these fragmented mitochondria into the extracellular space to promote osteoprogenitor differentiation and thus accelerate bone regeneration. (**E**) MSCs cocultured with neurons can be primed toward a status more suitable for neuroprotection due to cytoplasm exchange and mitochondrial transfer. (**F**) Active platelets transfer respiratory-competent mitochondria to MSCs, leading to the secretion of proangiogenic factors, which improves the wound-healing capacity of MSCs. (**G**) When co-culturing ESCs with SHED, mitochondria from SHED can translocate into ESCs through TNTs and induce ESCs towards neural crest differentiation. (**H**) In the bone marrow microenvironment, BMMSCs transfer mitochondria to HSCs when sensing bacterial infection, resulting in a metabolic shift in HSCs from glycolysis towards OXPHOS, so that HSCs can adapt to increasing energy demand for rapid expansion and granulopoiesis in response to infection. Schematic diagram created with Biorender

cerebral tissues [13, 89]. The enhancement of MSCs on angiogenesis can be explained in two ways. Firstly, MSCs exert their effects by delivering mitochondria to endothelial cells to promote the tube formation, resistance to oxidative stress, and growth factor secretion of the latter. EPCs-derived particles containing mitochondria have been shown to positively regulate angiogenesis with an elevated expression of vascular endothelial (VE)-cadherin in both normal brain endothelial cells and OGD-treated damaged brain endothelium. Uptake of mitochondria restored brain endothelial energetics with increased ATP production [90]. Concentrates derived from platelets are capable of decreasing apoptosis in endothelial cells subjected to H_2O_2-induced oxidative stress. When injected into murine cutaneous wounds, the platelet concentrates accelerated the rate of healing and intensified angiogenesis, as indicated by CD31 (namely platelet endothelial cell adhesion molecular) expression, compared to the non-treated group. This protective effect was partly mediated by platelet-derived mitochondria transferred to HUVECs. Internalization of mitochondria led to a significant upregulation in pathways associated with stress, anti-apoptosis and cellular proliferation in HUVECs [45]. When BMMSCs-derived mitochondria were cocultured with HUVECs, they were internalized and thus protected the HUVECs from oxidative stress-induced apoptosis and reduced production of ROS. HUVECs integrated with donor mitochondria exhibited enhanced tube formation, stem cell factor (SCF) secretion, ATP production and cell proliferation capability [91].

Secondly, MSCs internalized extra mitochondria exhibit improved capacity to support angiogenesis. When mitochondria isolated from platelets were internalized by ADSCs, mitochondria-potentiated ADSCs showed

enhanced secretion of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). After the modified ADSCs were grafted into mouse wounds, they were capable of triggering more angiogenesis-related marker expression, such as CD31, VE-cadherin, and VEGF. In addition, more endothelial cells were recruited at the injury site with improved wound vascularization and wound-healing capability [13].

Mitochondrial transfer promotes the pro-angiogenic potential of MSCs and enhances the angiogenesis of endothelial cells. The pro-healing and pro-angiogenic effect of mitochondrial transfer corroborates its prospective application in regenerative medicine.

Osteogenic/odontogenic differentiation

There are intricate cellular contacts in the bone marrow environment that regulate the fate of BMMSCs which closely correlate with bone metabolic homeostasis or dysfunction. The exchange of mitochondria may unveil an unprecedented realm of regulatory mechanisms. According to Suh et al., the morphological changes of mitochondria caused by fusion or fission could critically influence osteoblast maturation and osteogenesis. Specifically, osteogenic induction of osteoblasts triggers mitochondrial fragmentation and extracellular release of mitochondria, which acted on osteoprogenitor differentiation and bone regeneration positively [30]. Although the subsequent fate of excreted extracellular mitochondrial donuts has not been further discussed in this article, the role of mitochondrial transfer during osteogenesis can be anticipated. Macrophages are capable of delivering mitochondria to BMMSCs and contributing to the maintenance of bone homeostasis. In osteoporotic mice, this balance was disrupted by an elevated transfer of oxidatively damaged mitochondria to BMMSCs, which led to succinate accumulation, elevated ROS levels, and hypoxia-inducible factor1α (Hif-1α) activation. As a result, the uptake of damaged mitochondria stimulated the expression of pro-inflammatory genes, which in turn hindered osteogenic differentiation of BMMSCs and bone formation [92].

The metabolic shift during odontogenic differentiation of dental pulp stem cells (DPSCs) has been widely investigated recently. At an induction interval of 7 days, the energy supply of differentiating DPSCs undergoes a shift from glycolysis towards OXPHOS, indicating a requirement for mitochondrial respiration during odontogenesis [93]. In a study by Wang et al., the thermoplastic effect of Au nanoparticles was used to target mitochondria, so as to increase MMP levels, ATP production and promote the odontogenic differentiation potential of DPSCs. The study showed that the photothermal-driven DPSCs differentiation showed less induction interval without the addition of a conventional induction medium [94]. Healthy DPSCs can deliver their mitochondria to LPS-treated DPSCs in a contact-dependent manner both during co-culture and in murine pulp injury models. Mitochondrial transfer restored impaired mitochondrial function, and the fate of DPSCs towards proliferation and odontogenic differentiation was altered by mitochondrial transfer during this process as well [95].

Artificial mitochondria transplantation holds huge potential in modifying the osteogenic/odontogenic differentiation of stem cells. The study by Ma et al. investigated how the inhibitory effect on osteogenesis due to low matrix stiffness can be rescued by mitochondrial transfer. To be specific, a low-stiffness culture environment resulted in mitochondrial dysfunction in periodontal ligament stem cells (PDLSCs), as indicated by increased ROS generation and reduced ATP production. The uptake of mitochondria derived from PDLSCs cultured in favorable conditions has been shown to reverse this mitochondrial dysfunction as well as recover the osteogenic potential [80]. Autologous mitochondrial transfer of BMMSCs shows enhanced proliferation, migration, and osteogenic differentiation potential compared to BMMSCs without modification. When applying those mitochondria-recipient BMMSCs to bone defects, they performed better in wound healing than untreated BMMSCs [5]. The regulation of mitochondrial transfer over osteogenesis/odontogenesis has been gradually recognized, unveiling its potential in wound healing, repair, and regeneration of mineralized tissue.

Neural differentiation

Mitochondrial activity is crucial for neural differentiation of stem cells. During neuronal differentiation of stem cells from human exfoliated deciduous teeth (SHED), increased MMP and increased mtDNA could be detected. Inhibition of mitochondrial respiration and activity has resulted in inhibition of neuronal differentiation [96]. When co-cultured with human embryonic stem cells (ESCs), SHED were able to induce ESCs towards neural crest differentiation [97]. Until recently, it has been discovered that mitochondria from SHED can translocate into ESCs through TNTs during this induction process [84]. When in response to injury, mitochondria in embryonic hippocampal neurons go through a dynamic fusion-fission process and show a fragmented morphology, suggesting mitochondrial replenishment might be crucial for neuronal regrowth [98].

Intercellular mitochondrial transfer has been suggested to play a significant role in repairing ischemic and hemorrhagic injuries, rescuing SCI, alleviating neurotoxicity, and preventing neurodegeneration. Supplying fresh mitochondria isolated from cortical neurons to the injured hippocampal neurons significantly increased neurite re-growth as well as recovered neuronal function

in vitro [98]. One study cocultured Schwann cells (SCs) with hUCMSCs-derived mitochondria, the SCs internalized mitochondria and showed enhanced proliferation, migration, and respiratory capacity [61].

Overall, mitochondrial transfer exhibited high functionality to promote nerve regeneration, providing a novel regenerative strategy based on improving energy metabolism for neural repair.

Intercellular crosstalk

Mitochondrial transfer constitutes an important part of intercellular communications, leading to alterations in both donor and recipient cells. On the one hand, stem cells can fuse with adult somatic cells and promote the reprogramming of somatic cells back to a progenitor-like state. Both ADSCs and BMMSCs have been shown to reprogram mature cardiomyocytes through a partial cell-fusion process during coculture. Transfer of stem cell mitochondria into cardiomyocytes is essential for this reprogramming process since deletion of donor mtDNA leads to a decrease in cardiac hybrid cells as indicated by the downregulation of proliferation marker GATA binding protein 4 (GATA-4) [99]. On the other hand, stem cells cocultured with certain somatic cells can be primed toward a status more suitable for regeneration due to cytoplasm exchange and mitochondrial transfer. MSCs cultured with cerebral cortex neurons show more intensified neuroprotective potentials with elevated excretion of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) [100]. When co-cultivated with rat cardiomyocytes, human BMMSCs show expression of myosin, a specific human cardiac marker. Additionally, mitochondrial transfer from BMMSCs to cardiomyocytes has also been identified [101].

These studies have uncovered a previously unknown ability of mitochondrial transfer to modulate cell fate, offering new insights in reprogramming somatic cells and induction of pluripotent stem cells. Moreover, somatic cells regulate stem cells by exchanging mitochondria, organelles or partial cytoplasm, which can be used for priming stem cells before practical application in clinical settings. These findings have far-reaching implications for regenerative medicine and hold promise for advancing the field in novel ways.

Immunomodulation

In the bone marrow microenvironment, mitochondria from BMMSCs have been reported to transfer to HSCs in a contact-dependent manner within 2 h of sensing bacterial infection. Herein, mitochondrial transfer was shown to induce metabolic changes in HSCs, shifting cellular metabolism from glycolysis towards OXPHOS. This metabolic shift is responsible for the rapid expansion of HSCs and granulopoiesis in response to infection [29]. MSCs are able to donate mitochondria to recipient immune cells and modulate inflammatory reactions. From a study by Patricia et al., Th17 cells are capable of absorbing mitochondria from BMMSCs during coculture, leading to a decrease in IL-17 production and conversion toward T regulatory (Treg) cells. BMMSCs have been shown to exert an immunosuppressive effect mediated by mitochondrial transfer, which persists even when BMMSCs-derived mitochondria are artificially transplanted into Th17 cells [26].

When the dental pulp suffered from inflammation, intensified mitochondrial oxidative stress was detected in odontoblasts, which caused mitochondrial damage and led to the inevitable onset of mitophagy [102]. Healthy BMMSCs are capable of donating their mitochondria to infected odontoblasts via TNTs, thus reducing mitochondrial oxidative stress and preventing the pyroptosis of odontoblasts [103]. Intercellular mitochondrial transfer shows reparative potential in pulpitis and might be a therapeutic target for dental pulp repair. When cocultured DPSCs with LPS-pretreated DPSCs, mitochondria dynamically migrated toward adjacent injured DPSCs so that recipient cells can restore functionality with increased cellular ATP content, antioxidant levels, superoxide dismutase, and catalase activities [95].

MSCs-derived mitochondria have been shown to have immunomodulatory effects on a plethora of effector cells, including HSCs, macrophages, T cells and other somatic cells. This potential of immunomodulation is essential for the therapeutic application of mitochondrial transfer in tissue repair and regeneration.

Therapeutic application of mitochondrial transfer

The multifaceted regulatory effects of mitochondrial transfer on cellular activities have spurred interest in harnessing this biological mechanism for therapeutic applications. Initially, mitochondrial therapeutics were developed through microinjection of mitochondria into oocytes to address mtDNA mutation diseases in embryology. The discovery that cells can actively internalize mitochondria from both their extracellular environment and neighboring cells has driven researchers to establish postnatal mitochondrial transplantation strategies.

Isolation and purification of mitochondria

Mitochondria are extracted from lysed tissues or cells mainly through the following techniques: differential centrifugation, density gradient highspeed ultracentrifugation, filtration, commercial kit-based isolation, and magnetically labeled TOM22 antibodies for mitochondrial extraction (known as fractionated mitochondrial magnetic separation) [83, 104–107]. Isolation methods such as density gradient centrifugation and affinity purification by magnetic beads can yield mitochondria with

high purity, but are time-consuming and low in output. Differential centrifugation, filtration and commercial kit-based approach, which allow a rapid preparation, is characterized by high yield and low purity. Both differential centrifugation and filtration have been used in clinical trials [104, 108]. A less time-consuming method that yields mitochondria with high purity should be considered for future application in patients, as mitochondria can be directly used for subsequent transplantation after being separated from other cellular components.

Mitochondria microinjection

The feasibility and possibility of mitochondria transplantation in disease treatment were initially confirmed by mitochondria replacement therapy through microinjection. To combat maternally inherited mtDNA diseases, embryologists transplanted the nuclear genome from the oocyte with pathogenic mtDNA mutation to an enucleated oocyte from a healthy donor. The incorporated oocyte carrying healthy genome DNA and nonpathogenic mtDNA was further used for in vitro fertilization (IVF) [109]. During the process of cytoplasmic transplantation, active mitochondria were transplanted from a donor oocyte to a compromised recipient oocyte and were observed to pass on and function throughout embryonic development.

Apart from this mitochondrial replacement methodology, mitochondria microinjection has also been used to improve the outcome of IVF (Fig. 6A). Mitochondria were extracted from autologous ADSCs and microinjected into oocytes along with intracellular sperm injection in juvenile mice. Mitochondria potentiate zygotes and show improved embryonic development [110]. However, in a registered clinical trial that aimed to improve the pregnancy prognosis of infertile patients with premature ovarian aging, microinjection of mitochondria-derived from autologous egg precursor cells during sperm injection did not seem to exhibit significant improvement in embryo quality [111].

Mitochondrial applications in fertilization and embryology are especially meaningful for validating the practicality of subsequent postnatal exploitation in mitochondrial transplantation. Upsurging academic attention has been put on the therapeutic application of mitochondrial transfer. One approach is to potentiate target cells with purified, functional mitochondria before administering them to patients, considering mitochondrial transplantation as an additional procedure for cell manufacturing and processing to improve cellular potential.

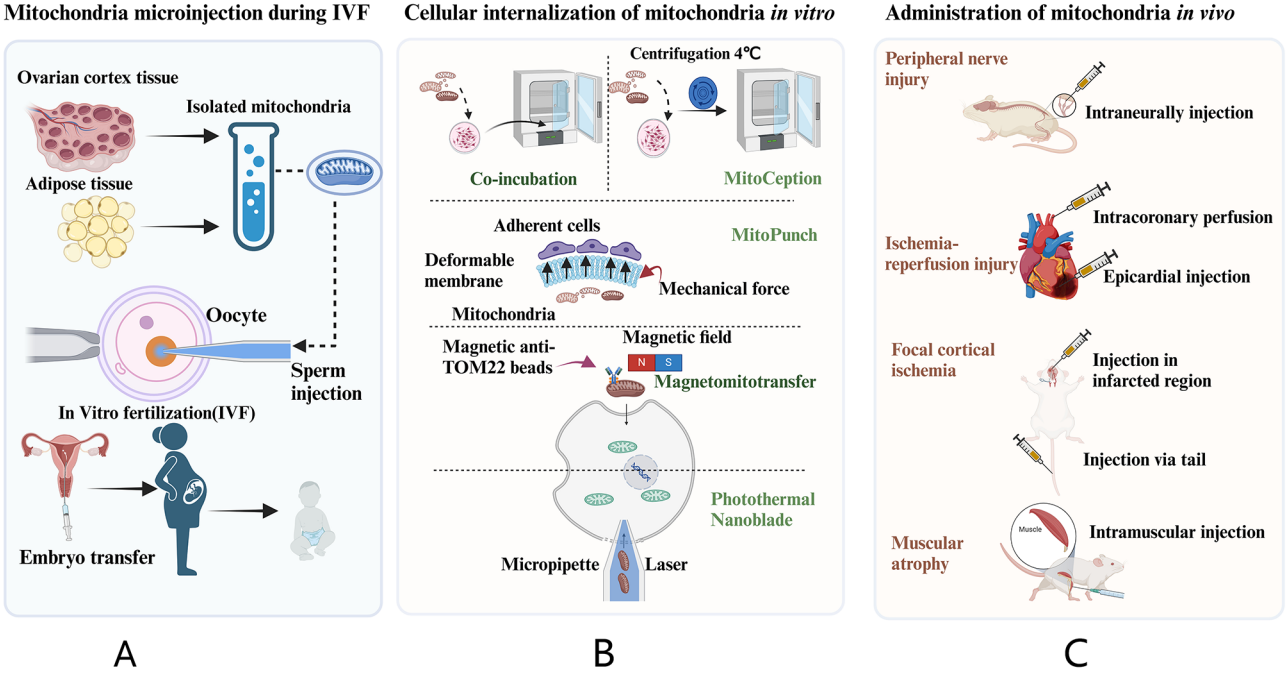


Fig. 6 Illustrations for the Therapeutic applications of mitochondrial transfer. (**A**) Mitochondria extracted from ovarian cortex tissue or adipose tissue are microinjected into oocytes along with intracellular sperm injection so as to improve the outcome of IVF and assisted reproduction. IVF, in vitro fertilization. (**B**) Graphic illustration of physical strategies to promote internalization of exogenous mitochondria into recipient cells. MitoCeption: centrifugation and thermic shock followed by coincubation. MitoPunch: mechanical force generated by a specific device pump mitochondria suspension into the monolayer cell culture to promote internalization. Magnetomitotransfer: employs magnetic anti-TOM22 beads to label mitochondria and carries them into host cells with the support of a magnetic field. Photothermal nanoblade: applies laser nanoblade to break through cell membrane and transfer mitochondria through a micropipette. (**C**) Therapeutic applications of mitochondria through either systemic or local administration in disease models such as peripheral nerve injury, ischemia-reperfusion injury, focal cortical ischemia and muscle atrophy, etc. Schematic diagram created with Biorender

One example is to apply maternal mitochondria for the enrichment of HSCs isolated from pediatric patients diagnosed with mtDNA deletion syndromes, and subsequently transfused the augmented HSCs back to patients intravenously [108]. Another methodology involves the direct administration of mitochondria or EVs containing mitochondria to the bloodstream or injury site, such as the treatment of ischemia-reperfusion injury with autogenous healthy mitochondria through epicardial injection or coronary catheter delivery [19, 20].

Approaches to promote internalization during mitochondria transplantation

Ongoing efforts are being made to achieve targeted delivery of mitochondria *in vitro.* The cellular uptake of mitochondria can occur through circulation or local injection in a specific tissue site, suggesting that mitochondrial transfer could also be achieved through coincubation with recipient cells alone. This possibility has been validated in many cells, including cardiomyocytes, HUVECs, SCs, and ADSCs [13, 61, 65, 112]. However, solely coincubation is incapable of incorporating a high rate of foreign mitochondria. Researchers have developed numerous approaches to increase transfer efficiency (Fig. 6B). One option is centrifugation and thermic shock before coincubation, which is known as MitoCeption. Specifically, the coincubation of recipient cells and mitochondria suspension go through two cycles of centrifugation at 4 °C [113]. Besides, there is another approach known as magnetomitotransfer. This is achieved by first labeling mitochondria with magnetic antibody beads and then exposing the isolated mitochondria and recipient cells to a magnetic plate to facilitate internalization [114]. MitoPunch is another option for applying mechanical force to facilitate mitochondrial transfer in a high throughput way. Pressures generated by a sophisticated device pump mitochondria suspension into the monolayer cell culture and the force is simultaneously transmitted to cells to promote mitochondrial internalization [115]. There is also a report on using photothermal nanoblade to transfer isolated mitochondria into cells. This technique involves creating a transient opening in the cellular membrane to allow for the absorption of the mitochondria cargo. Although mitochondrial transfer through this approach is more efficient than cell fusion, the throughput is still low [116]. Physical approaches have a high risk of damage to target cells and mitochondria; furthermore, they cannot be performed in vivo.

Besides transplanting mitochondria through physical forces, conjugating mitochondria with coatings or developing carriers to embed mitochondria have also been put forward. One such approach is to conjugate isolated mitochondria to the cell-penetrating peptides (such as Pep-1, transactivator of transcription peptides) or triphenylphosphonium polymer to facilitate the entry into recipient cells. As a result, mitochondria conjugated with coatings had better delivery efficiency than naked mitochondria [117–119]. Lin et al. developed a carrier for efficient delivery of mitochondria by incorporating glycoprotein G of vesicular stomatitis virus into plasma membrane vesicles. Their results showed that this method was able to restore the shape and MMP of mitochondria, resulting in a two-fold increase in transferred mitochondria in cultured HeLa cells devoid of functional mitochondria compared to non-treated samples [120]. Another research team proposed a method of delivering free mitochondria using fusogenic liposomes, where the encapsulated mitochondria maintained their structural stability and viability, with minimal cytochrome C leakage. Moreover, unlike naked mitochondria, the encapsulated mitochondria enter recipient cells through membrane fusion and are absorbed through endocytosis. The team found that a higher amount of mitochondria accumulated intracellularly due to liposome encapsulation [16]. Despite the above attempts, further efforts are necessary to develop novel strategies that can achieve high efficacy and efficiency in mitochondria transplantation. All of the aforementioned strategies have improved the metabolic activity and mitochondrial function of recipient cells to some degree; however, they cannot be applied in vivo due to their inability to target specific cells and avoid being scavenged by the host.

Therapeutic applications of mitochondria transplantation-potentiated stem cells

There are preclinical studies utilizing exogenous mitochondria-potentiated stem cells in disease treatment in animal models such as skin wound, bone defects, and lung injury [12, 14, 27]. Specifically, MSCs internalized autogenous or allogeneic mitochondria have been implanted into murine injury sites such as cutaneous wounds and bone defects. Better tissue repair and injury healing outcomes have been observed in mitochondria-transplanted group than non-preconditioned MSCs. Limitations of directly applying mitochondria-recipient MSCs in vivo are explicit. Firstly, the interactions between implanted MSCs and other cells such as immune cells, endothelial cells are largely unknown. Secondly, the quantity of available autogenous MSCs is limited, which could in turn impede the clinical application of mitochondria-enriched MSCs from the same patient.

Clinical research of this field is in the early stages of exploration. Jacoby et al. extracted mitochondria from human placenta tissue and transferred them to HSPCs from patients with mtDNA deletion or mutation syndromes (namely Pearson syndrome and Leigh syndrome, respectively). Mitochondrial augmentation of HSPCs did not alter their in vitro ability to generate hematopoietic

colonies. When transplanting modified HSPCs into an immunocompromised murine model intravenously, six-month durability in the engraftment of human hematopoietic cells in the bone marrow was noted [83]. Further clinical study was carried out on paediatric patients with Pearson syndrome, in which they received a transfusion of autologous HSPCs carrying additional mitochondria, isolated from maternal peripheral blood mononuclear cells (PBMCs). Following treatment, a decrease in heteroplasmy and an increase in mtDNA content of peripheral blood cells were observed. Moreover, patients with very low body weight before treatment showed improvements in aerobic function and an increase in body weight [108]. The application of autogenous HSPCs significantly reduced immune rejection in this clinical trial. Moreover, donor mitochondria are maternally derived, which mitigated mitochondrial heteroplasmy in recipient HSPCs. Although the underlying mechanism of how exogenous mitochondria-modified HSPCs mitigating inherited mitochondria diseases remains unknown, this attempt is still significant for future progress since modified cells demonstrate improved performance.

Therapeutic applications of mitochondria through systemic and local administration

Another approach is the direct administration of free mitochondria or mitochondria-containing EVs to the circulation or injury site (Fig. 6C). Autologous or allogenic mitochondria are frequently applied in indications including but not limited to cardiovascular diseases, injuries to central or peripheral nervous system, neurodegenerative diseases and metabolic syndromes etc [17, 82, 121, 122]. Both local and systemic injections of mitochondria have shown therapeutic effects by restoring mitochondrial functions and bioenergetics. Intriguingly, based on the detection of fluorescence-labeled mitochondria, systemically administered mitochondria derived from human hepatoma cells demonstrate the capability to penetrate physiological barriers and distribute within brain, heart, liver, kidney and muscle tissues in mice [123]. Another study conducted by Zhu et al. reported that mitochondria derived from femoral artery smooth muscle cells were trapped in rat pulmonary arteries after intravenous injection, with minimal amounts of exogenous mitochondria found in kidney, liver and spleen. The researchers speculated that mitochondria were unable to cross systemic boundaries due to their size [124]. These studies suggest that the systemic administration of mitochondria lacks specificity. Compared to the application of mitochondria-potentiated MSCs, direct evidence of mitochondrial transplantation leading to these favorable results is still lacking. The internalization of mitochondria in targeted cells and the causal link between mitochondrial engulfment and detected functional improvement require further investigation.

Clinical trials have been carried out in paediatric patients who suffered from ischemia-reperfusion injury and required central extracorporeal membrane oxygenation (ECMO) support after cardiac surgery. Healthy autologous mitochondria harvested from non-ischemic skeletal muscle were used for epicardial injection. Although a randomized controlled study design was not employed in this study, recruited patients did not have adverse short-term complications due to mitochondrial injection, and all demonstrated improvement in ventricular function within several days after treatment [19]. Another clinical study utilized allogeneic platelets to extract mitochondria and transfused them through the coronary guiding catheter to patients suffering from acute myocardial infarction. No significant difference in adverse outcomes was observed between the conventional treatment group and the mitochondrial transfer group [20]. These studies confirmed that the administration of mitochondria, either autologous or allogeneic, can be well tolerated by recipients. In addition, naked mitochondria and EVs-encapsulated mitochondria are naturally abundant in blood or blood products, providing conclusive evidence that autologous or heterologous mitochondria can be safely applied systemically or topically to some extent [66].

Ongoing clinical trials of mitochondrial therapeutics

Currently, mitochondrial therapies are being used in clinical research for a wider range of applications, including cardiovascular diseases, mitochondrial diseases myelodysplastic syndromes (MDS), refractory polymyositis, dermatomyositis and repeated IVF failures according to https://clinicaltrials.gov/. The participants are distri buted across a diverse range of ages, including pediatric to elderly individuals. Meanwhile, the sources of tissues and cells for mitochondrial isolation are not limited to autologous origins, allogeneic hUCMSCs and placental tissue-derived mitochondria are also being utilized. Investigators are exploring diverse approaches to administer mitochondrial treatments, which include methods such as local injection into the injury site, intravenous injections, and microinjections during IVF etc. (Table 2). These clinical studies suggest a broad application prospect of mitochondrial transfer in the treatment of diseases. Ongoing clinical trials are expanding the scope of mitochondrial therapy applications, uncovering a broader perspective for research. With extended observation period and application of heterologous mitochondria, these clinical trials may provide us with a deeper understanding of the immunocompatibility of mitochondrial therapy. Rigorous prospective clinical trials with long observation period are necessary to

Table 2 On-going clinical trials of mitochondrial therapeutics

Target Disease	Patient recruitment	Mitochondrial source	Delivery approach	Observation period	Outcome measurement	Location/Country	ClinicalTrials.gov ID
Cerebral ischemia	18–85 years old	Healthy autologous mitochondria from the muscle tissue adjacent to the surgical access site	Endovascular via micro-catheter during reperfusion	1 week	Severe adverse events; Reduction of infarct volume post-mitochondrial infusion	Washinton DC/USA	NCT04998357
Mitochondrial diseases	Women with confirmed mtDNA mutation	Not mentioned	Injection with ICSI	18 months	Neurodevelopmental, assessment of newborns	Newcastle/UK	NCT04113447
Pearson Syndrome	Paediatric patients (1–18 years old)	Allogeneic placental-derived mitochondria from healthy donors	MNV-201	12 months	Treatment-related adverse events	Ramat Gan/Israel	NCT06017869
Mitochondrial disease	Paediatric Patients (4–18 years old)	Allogeneic placental-derived mitochondria from healthy donors	MNV-201	1 month	Treatment-related adverse events	Ramat Gan/Israel	NCT04548843
Myocardial ischemia	Paediatric cardiology patients ≤ 18 years on ECMO	Biopsies collected from the exposed skeletal muscle of the chest wall	Local injection to ischemic area or systemic administration	1 month	Severe adverse events	Boston/USA	NCT02851758
Heart failure	35–80 years old	Allogeneic hUCMSCs	Intracoronary and intra-myocardial injection of exosomes and mitochondria	3 months	Left ventricle ejection fraction and allergic reactions	Tehran/Iran	NCT05669144
Repeated IVF Failure	Female patients ≥ 35 years old	Autologous USCs	Injection with ICSI	12 months after embryo transfer	Live birth rate	Beijing/China	NCT06020742
MDS	Patients ≥ 18 years old	Allogeneic placental-derived mitochondria from healthy donors	MNV-201	12 months	Occurrence of treatment-related adverse events	Jerusalem/Israel	NCT06465160
Refractory polymyositis or dermatomyositis	Patients ≥ 19 years old	Allogeneic hUCMSCs	Intravenously injection	3 months	Toxicity and efficacy	Seoul/South Korea	NCT04976140

Abbreviations: ICSI, intracytoplasmic sperm injection; MNV-201, a cell therapy that isolates participant's autologous CD34+hematopoietic stem and progenitor cells then enriches them with allogeneic placental mitochondria before transfusing back; ECMO, extracorporeal membrane oxygenation; hUCMSCs, human umbilical cord-derived mesenchymal stem cells; IVF, in vitro fertilization; USCs, urine derived stem cells; MDS, myelodysplastic syndromes

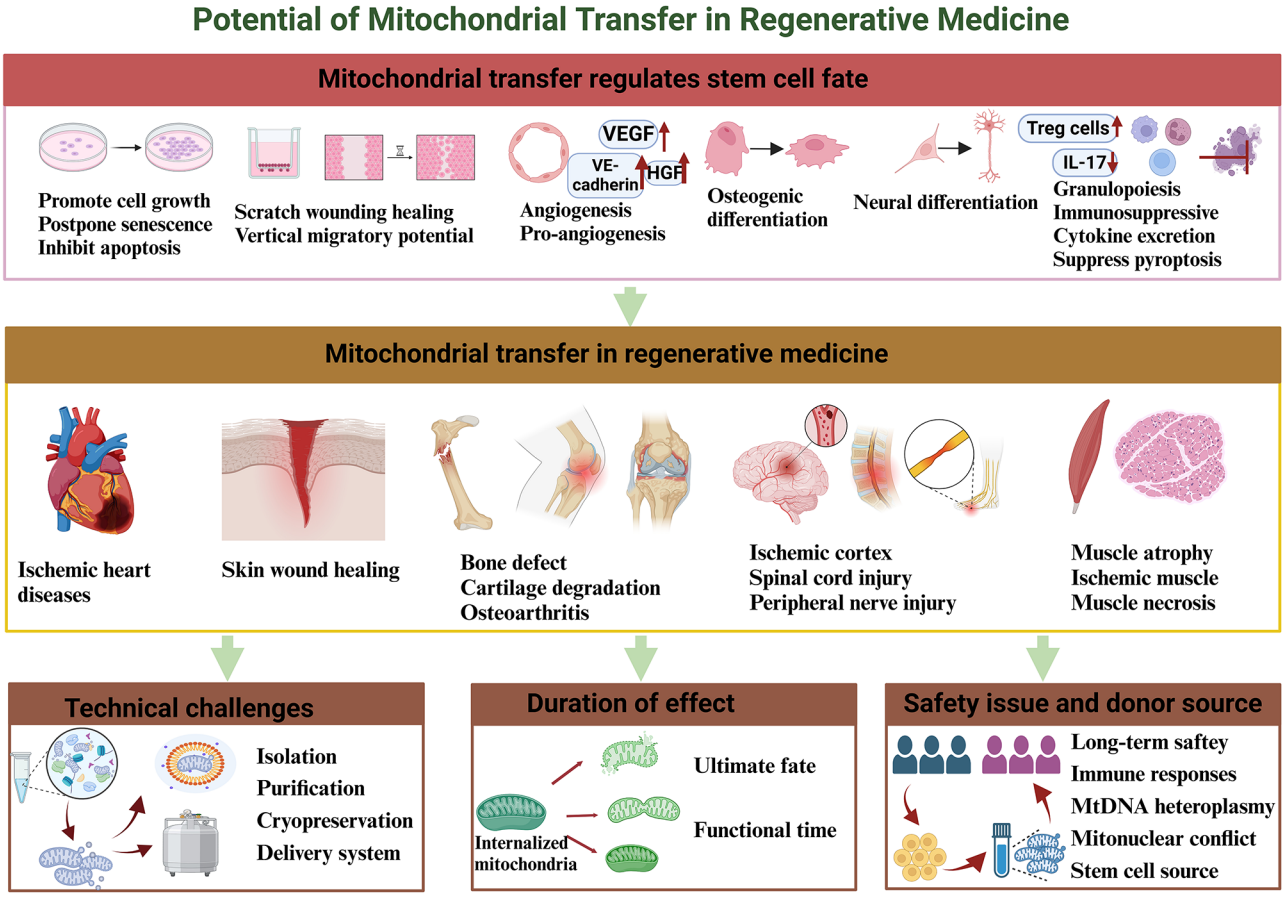


Fig. 7 Potential of Mitochondrial Transfer in Regenerative Medicine. Mitochondrial transfer regulates multiple cellular processes regarding proliferation, viability, migration, differentiation, and immunomodulation in various cell types and species. The in vitro findings of mitochondrial transfer regulation over stem cell fate lay solid foundations for its in vivo applications in the revitalization of ischemic diseases, healing of defects, regeneration of muscular or skeletal tissue, and repair of nerve injury. There are issues worth noting before in-depth clinical application of mitochondrial therapies. For instance, standardized protocols for the extraction, purification, storage, delivery of mitochondria need to be established. The fate of mitochondria and the functional time after internalization is uncertain. The long-term safety and immune reactions of host after mitochondria transplantation remain to be solved. Schematic diagram created with Biorender

comprehensively evaluate the safety and effectiveness, as well as to formulate standard mitochondrial therapy protocols for each disease.

Potential of mitochondrial transfer therapeutics in regenerative medicine

Mitochondrial transfer is capable of promoting cellular potentials regarding proliferation, migration, immunoregulation, angiogenesis, and differentiation (Fig. 7). Mitochondrial transplantation has been shown to enhance stem cell properties at multiple levels, leading to a growing body of research focused on utilizing this approach to engineer stem cells for tissue regeneration [13, 15, 41]. Notably, studies have demonstrated that mitochondria derived from MSCs can independently promote tissue regeneration, even in the absence of intact MSCs. This phenomenon mirrors the regenerative effects of MSCs themselves, thereby establishing a foundation for a novel paradigm in stem cell-free regenerative therapies [15, 17, 18, 125]. Research has been made in applying mitochondrial transplantation for the repair, revitalization or regeneration of the myocardial tissue, skin, bone, nerves, blood vessels and muscles.

Cardiovascular tissue revitalization

The cardioprotective potential of stem cell-based mitochondrial transfer in treating ischemic heart diseases largely relies on their ability to promote the revitalization and angiogenesis of ischemic regions. When coculturing BMMSCs with HUVECs under OGD conditions, mitochondria migrate from BMMSCs to HUVECs in a unidirectional manner, rescuing HUVECs from apoptosis and promoting tube formation by restoring aerobic respiration [74]. This phenomenon of mitochondria exchange arises during the cocultivation of BMMSCs and VSMCs as well [8]. Intercellular mitochondrial transfer between

endothelial cells and MSCs lays the foundations for the pro-angiogenic effects of mitochondrial therapies in cardiac revitalization.

The intercellular mitochondria exchange between MSCs and cardiomyocytes during coculture has also been widely investigated [101, 126, 127]. Through donating mitochondria, cardiomyocytes potentiate MSCs toward cardiac differentiation by promoting myosin expression [101], increase the secretion of pro-angiogenic factors, and boost chemotaxis [126]. In contrast, mitochondria from healthy MSCs can rescue damaged cardiomyocytes by restoring their MMP and resistance against apoptosis [127], improving their oxygen consumption, ATP production, and metabolic status [128]. Preconditioning MSCs with mitochondria extracted from cardiomyocytes also improves the regenerative potential of MSCs, as indicated by intensified proliferation, pro-angiogenic, immunomodulatory, and anti-fibrotic properties [56].

Mitochondrial administration in the treatment of ischemic heart diseases has been applied in multiple animal models such as rabbits, rats, mice, and piglets [15, 72, 129, 130]. Respiration-competent mitochondria, isolated from healthy tissue and then injected locally into the ischemic zone or perfused through coronary catheter, significantly enhance postischemic functional recovery and cellular viability [72, 129, 130]. The study conducted by Jin et al. applied hUCMSCs-derived mitochondria in the rescuing of murine heart attack through intravenous injection. They found transplantation of MSC-derived mitochondria could preserve cardiac function and prevent cardiomyocyte apoptosis by restoring ATP production and inhibiting excessive autophagy [15]. The therapeutic effects were achieved with mitochondria solely, without MSCs, indicating the feasibility of a cell-free approach as a potential alternative to MSC-based regenerative therapies.

The first clinical application of mitochondria transplantation was carried out on children with myocardial ischemia–reperfusion injury [19]. Another clinical trial from Iran evaluated the efficacy of platelet-derived mitochondria transplantation to adult patients with cardiac ischemia [20]. These two studies both show safety and efficacy in mitochondrial administration in cardiac diseases. In clinical trials, it is still early for the application of MSC-derived mitochondria, likely due to the challenges in finding safe and sufficient donor MSCs.

Above in vitro experiments, animal studies, and clinical trials validate the feasibility and efficacy of mitochondria transplantation in treating cardiomyopathies. Based on the outcomes of extensive preclinical studies, mitochondrial transfer between MSCs and cardiomyocytes or endothelial cells has demonstrated the ability to promote pro-angiogenesis, revitalization, and shows protective effects on ischemic cardiac tissue. It is worth exploring the possibility of applying MSCs, either autogenous or allogeneic, as mitochondrial donor in cardiovascular tissue revitalization to expand the donor cell source. Future research needs to establish rigorous and concrete strategies before considering clinical applications with a broad range of indications.

Skin tissue regeneration

The wound healing and skin repair process involves hemostasis, regulation of immune response, stem cell proliferation, migration, and angiogenesis. The engraftment of adipose-derived stem cells (ADSCs) potentiated with platelet-derived mitochondria in full-thickness cutaneous wounds showed a higher wound healing rate than grafting ADSCs with no treatment, confirming that mitochondria from platelets promote the therapeutic efficacy of ADSCs. Further investigation excluded the involvement of platelet-derived mitochondria in stimulating differentiation, immunoregulation, proliferation or cytoprotection effects on ADSCs. However, mitochondria from platelets enhance pro-angiogenic potential through remodeling the metabolic status of ADSCs, leading to upregulation in VEGF and HGF secretion, which resulted in more favorable wound healing effects [13]. In a rat skin defect model, implantation of ADSCs carrying additional allogenic mitochondria resulted in improved skin repair at both morphological and functional levels compared to ADSCs without mitochondrial transplantation. The mitochondria-transferred ADSCs exhibit an increase in ATP production, enhanced proliferation and migration ability, highlighting the potential of mitochondria to enhance the regenerative properties of ADSCs for skin repair [41].

Hard tissue regeneration

Healthy HSPCs can transfer mitochondria to BMMSCs, leading to a reconstituted hematopoietic system of bone marrow and supporting the metabolic recovery of the stromal microenvironment after irradiation [41]. Pretransplanting BMMSCs with autogenous mitochondria has been shown to enhance the regenerative potential of BMMSCs on bone defect healing in situ, with more intensified new bone formation observed [12]. BMMSCs also showed protective effects on reducing cartilage degeneration through mitochondrial transfer. When coculturing BMMSCs with chondrocytes derived from osteoarthritis, mitochondrial transfer from BMMSCs to chondrocytes was detected. This transfer resulted in improved mitochondrial function, enhanced cell proliferation, and decreased apoptosis in chondrocytes [131]. In the murine osteoarthritis model, local injection of MSCs-derived mitochondria showed a therapeutic regenerative effect. This was evidenced by promoted tissue regeneration, including the recovery of cartilage and subchondral

bone, following intra-articular injection of liposomes-coated mitochondria [16].

The underlying role of mitochondrial transfer in the regeneration of dental hard tissues has also been gradually recognized, suggesting its potential application in dental regenerative therapies. In a rat dental pulp injury model, healthy DPSCs were added to the exposed dental pulp pretreated with LPS to imitate the inflammatory state. Apparent mitochondrial transfer and a significant increase in reparative dentin formation were observed in the exogenous DPSCs treatment group compared to groups without DPSCs treatment [95]. Wang et al. found that utilizing BMMSCs as donor cells to donate mitochondria to odontoblasts could protect odontoblasts against pulpitis by reducing inflammation-induced pyroptosis of odontoblasts [103]. The role of mitochondrial transfer in alleviating cell death and pathogenesis in dental pulp damage implies a new mitochondrial therapeutic strategy for dental pulp repair and regeneration, showcasing its promising potential in improving dental health outcomes.

Neuronal regeneration

Mitochondrial dysfunction and abnormalities are closely associated with neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, cerebral ischemic stroke, traumatic brain injury, SCI and peripheral nerve damage and other conditions. Mitochondrial therapy has emerged as a novel therapeutic approach in neuronal repair and regeneration [132].

A number of researchers directly injected purified mitochondria at the affected site. When BMMSCs-derived mitochondria were intraneurally injected into a sciatic nerve crush injury model, mitochondria could be absorbed by neurons, activated transcription initiation regions of the activating transcription factor 3(*Atf3*) gene through ROS accumulation, further promoted the expression of downstream regeneration-associated genes and facilitated axon regeneration [125]. The same effects in locomotor function recovery were acquired when mitochondria were injected into the injured spinal cord [17]. Another team used mitochondria isolated from hUCMSCs and loaded them with acellular nerve allografts (ANAs) in the recovery of sciatic nerve injuries. The additional mitochondria in ANAs showed more satisfactory regenerative results than merely ANAs graft, as indicated by more rapid axonal extension, myelin sheath formation, and higher levels of vascular regeneration in the proximal region [61]. In mice with ischemic cortex, signs of activated neurogenesis were observed when mitochondria extracted from murine liver tissue were administered via the tail vein or to the injury site (in situ). Increased 5-bromo-2′-deoxyuridine ($BrdU^+$) doublecortin (DCX^+) /$BrdU^+$ cell ratio and expression of pro-neurogenic factors, such as signal transducer and activator of transcription 3 (STAT3), Nestin, cyclin D1 (CCND1), C-MYC, suggested that exogenous mitochondria have rescued focal ischemia and promoted neurogenesis [122]. Zhang et al. applied autologous mitochondria derived from the pectoralis major muscle to treat ischemic injury with middle cerebral artery occlusion. Isolated mitochondria were subject to intracerebroventricular injection. According to their results, the distribution of mitochondria in cerebral ischemic areas was detected 24 h post-injection, decreased infarction region and neurological deficits were identified, accompanied by increased doublecortin expression, which indicated growing neuroblast progenitor cells and enhanced neurogenesis [133]. These studies indicate that applying mitochondria in injured regions provides improved regenerative capacity both in the peripheral nervous system and the central nervous system.

Another approach is to administer free mitochondria intravenously. In a murine model, fluorescence-labeled mitochondria were detected in brain, and improved locomotor activity was observed. However, the delivery efficiency of mitochondria is relatively low, requiring a large quantity to act on damaged regions [123]. This particularly challenging for injuries in the central nervous system, where the blocking effect of blood brain barrier must be taken into consideration.

Muscle regeneration

Kim et al. preconditioned muscle cells with dexamethasone to imitate atrophy. They found that when muscle cells internalized intact mitochondria isolated from hUCMSCs, there was a significant increase in cell proliferation and ATP production. Additionally, MMP levels, which had been compromised by atrophy, were restored and mitochondrial ROS content return to normal levels. Consequently, the engulfment of additional mitochondria led to the blocking of the AMPK/forkhead box O3 signaling pathway, which is involved in atrophy [134]. This team further investigated the in vivo effects of locally administered hUCMSCs-derived mitochondria by injecting them into the soleus muscles of a rat muscle atrophy model. Mitochondria significantly improved the muscle mass, muscle fiber content, and muscle-specific marker expression in atrophic muscles [18]. Likewise, the direct injection of mitochondria isolated from healthy muscle tissue into murine ischemic limb successfully ameliorates the effects caused by ischemia-reperfusion injury, as evidenced by the decreased infarcted muscle areas and improved function recovery observed in the mitochondria-treated group [135]. In a rat model with innate skeletal muscle mitochondrial dysfunction, allogeneic mitochondria derived from rats that underwent endurance training were directly injected into hindlimb

muscles of recipient rats. While no significant enhancement in exercise indices was observed, improved mitochondrial function was detected in the intervention group [136]. In addition to local administration of mitochondria, another group delivered mitochondria systemically via tail vein to rescue barium chloride-induced gastrocnemius muscle necrosis. Mitochondria have been found to preferentially enter injured cells and improve the rate of muscle fiber repair, as well as the restoration of muscle function [137]. The administration of exogenous mitochondria in the treatment of muscular diseases is a promising approach to enhance muscle regeneration and promote recovery of muscular function.

With limited evidence on their internalization by host cells so far, it is reasonable to infer that these extracellular mitochondria might act more like mediators in signaling pathways that regulate tissue revitalization. Further research is needed to determine whether these free mitochondria are absorbed by cells, which types of cells they enter, and the pathways through which they function [138]. From what has been discussed above, mitochondrial transfer shows promising prospects in modifying the properties of stem cells, altering their fate and reshaping tissue microenvironment to support regeneration. However, the application of mitochondrial transfer in regenerative medicine is still in its infancy. Further investigations are needed to explore how the beneficial effects of mitochondrial transfer or transplantation can be harnessed in this field.

Current challenges in mitochondrial transfer therapeutics

According to previous research, the role of stem cells in injury repair and regeneration can be achieved through the following pathways: direct cell-cell interaction, paracrine activity, differentiation into somatic cells to replace injured cells, and the release of molecules supported by microvesicles [139]. The mechanism of mitochondrial transfer will represent a novel mode of action for stem cells in tissue regeneration [41, 140]. It is noteworthy that mitochondrial transfer from transplanted stem cells to host hepatocytes has been detected in vivo during splenic injection of human BMMSCs in a murine steatohepatitis model [141]. The internalization of additional mitochondria has modified various cellular properties of the target cells, including proliferation, differentiation, migration, immunomodulatory reactions, metabolism, senescence, and stress response. Compared to other stem cell-based therapies, mitochondria transplantation offers low immunogenicity and tumorigenesis risks. Additionally, it can achieve high quantities through the proliferation and amplification of stem cells. No inflammation or any other local or systemic complications have been reported following mitochondria transplantation treatment. While extensive research has been conducted on the therapeutic potential of mitochondrial transfer, several questions remain to be answered before effective clinical application can be realized.

Technical challenges

Technical predicaments are one of the main challenges in applying isolated mitochondria in regenerative medicine. The first challenge lies in extracting intact, purified, and viable mitochondria that have therapeutic properties rather than being pathogenic, since dysfunctional mitochondria may trigger a systemic or tissue-specific inflammatory response [55, 142]. Therefore, a suitable, stable and rapid isolation method that warrants further investigation for mitochondria transplantation is urgently needed [143].

Another technical difficulty is about long-term storage and maintaining the functionality and activity of mitochondria after cryopreservation. Isolated mitochondria can be kept on ice for approximately 1 h without undermining their transplantation efficiency [144]. Mitochondrial outer membrane integrity will be impaired when cryopreserved at − 80 °C. The cryoprotectants such as dimethyl sulfoxide (DMSO) and trehalose are capable of maintaining membrane integrity, as well as retaining most of the biological features of mitochondria [145, 146]. Optimized mitochondrial preservation methods will extend the time-frame required for mitochondrial transplantation and maximize the preservation of mitochondrial functions for therapeutic purposes.

The repair and regeneration of tissue injury will benefit from the targeted delivery of mitochondria into specific cells, tissues or organs. The development of a delivery system that enables stable mitochondrial release that targets specific cells or organs should be considered [147]. The cellular internalization of exogenous mitochondria varies significantly among different recipient cell types depending on the source of mitochondria. Firstly, the endocytic pathways involved in this process are distinct. For instance, cardiomyocytes engulf mitochondria from human uterine endothelial-derived MSCs through micropinocytosis independent of actin [58], but internalize autologous mitochondria in an actin-dependent manner [148]. Secondly, the delivery rate of exogenous mitochondria varies under diverse cellular status. Cells under stresses exhibit higher efficiency in mitochondrial transfer compared to cells in normal condition. Thirdly, further in-depth research is needed to determine the definitive mechanistic route for how mitochondria enter tissues from the bloodstream, instead of being entrapped or blocked after systemic administration [144]. We still cannot decipher why the distribution of systemically applied mitochondria appear in various tissues [123] or only in one specific tissue [124]. One research team has

developed a polypeptide (PEP)-triphenylphosphonium cations (TPP+)-mitochondrial compound to promote the selective mitochondrial delivery into ischemic myocardial infracted region, while intravenous injection of pure mitochondria would otherwise elicit no therapeutic effects on myocardial injury. According to them, this compound can effectively sense ischemic tissues, accelerate mitochondria translocation into cardiomyocytes and ameliorate tissue injury [149]. Despite the promising results of this study, the targeted delivery of mitochondria into specific cells, tissues or organs under diverse contexts is still complex and challenging.

Duration of effect

Another issue to be addressed is the functional time of internalized mitochondria. Despite emerging evidence on the effect of mitochondria injection in rescuing cardiac ischemia, there has been research indicating it only exhibits short-term bioenergetics enhancement, regardless of autologous, non-autologous, or interspecies mitochondrial transplantation [112]. For in vivo study, the fluorescence of mitochondrial labelling is detectable after 7 days of local injection in the cardiac infarction site, and the amount of donor mtDNA can be detected 14 days postinfarction but undetectable after 28 days [91]. When in the context of coculture, one study found transferred mitochondria with fluorescence labelling overlapped with host lysosomes or excreted to EVs 8 days post-transplantation, suggesting exogenous mitochondria go through lysosomal degradation or extracellular excretion eventually [150]. Another study evaluated the effect of functional mitochondria delivered to fibroblasts that were derived from individuals with MERRF syndrome (with mutations of mtDNA). The study found that the effects of mitochondria delivery lasted for a minimum of 21 days, with a sustained increase in MMP and a reduction in ROS. Moreover, more intensified mitochondrial biogenesis was observed in groups with better delivery efficiency post 15 days of treatment [117]. When transplanting MSCs-derived mitochondria into endothelial cells, the exogenous mtDNA was undetectable after 7 days. It is not the functional mitochondria, but the degradation of mitochondria that provoked mitophagy and subsequent mitochondrial biogenesis that enabled cellular capacity improvement [65]. Based on current evidence, it is difficult to determine the ultimate fate of transplanted mitochondria. It is unclear whether heterogeneous mitochondria will maintain their viability, stimulate the generation of new mitochondria or undergo degradation or functional silencing once they are absorbed by recipient cells of different types.

Safety and donor sources

Lastly, while no adverse outcomes have been reported so far, the long-term effects and safety of mitochondria administration still require further investigation. So far clinical research have exploited autogenous or maternal mitochondria that are homologous, the mitochondrial heteroplasmy levels are minimized [143]. This hugely limits the application scope of mitochondria transplantation. If mitochondrial transfer is to become a routine and stable therapeutic approach, stem cells originated from different cell types and subjects should be considered for mitochondrial extraction. If this happens, mtDNA heteroplasmy due to combination of two mtDNA haplotypes can become a potential factor leading to cellular aberrations and warrants further in-depth research in the future [151]. While mitochondria are low in immunogenicity due to their lack of surface antigens compared with viable stem cells, they have their unique concerns. One such concern is the possibility of mitonuclear conflict. Specifically, the introduction of exogenous mtDNA copies with different genetic profiles could disrupt the existing balance between mtDNA and nuclear DNA in recipient cells [143]. As undifferentiated stem cells are considered the most ideal candidates for mitochondrial donation, ethical issues, species origin and cell types must be taken into consideration when choosing a donor source [152].

Conclusions

The research of mitochondrial transfer in stem cells and regenerative medicine is currently at a thriving stage, with multiple ongoing studies exploring its application in various cell types, organ systems and diseases. The development of exogenous mitochondrial transplantation has broadened the scope of stem cell therapy, allowing for another cellular derivative to be used in modifying stem cell potential for therapeutic purposes.

This review provides a thorough understanding of mitochondrial transfer in the orchestration of stem cell fate, regarding self-renewal, proliferation, differentiation and intercellular crosstalk. It underscores the promising potential of mitochondrial transfer in the field of regenerative medicine, highlighting its importance for advancing treatment strategies. Further research is needed to fully investigate the methodologies to promote mitochondrial internalization, the ultimate fate of absorbed mitochondria, as well as technical challenges such as extraction, cryopreservation, and stable delivery of mitochondria with less off-target effects. The duration of effects and potential long-term responses based on specific cell, organs, tissues and diseases still require thorough study.

Whether to choose mitochondrial augmentation to enhance the cellular performance of stem cells before transplantation or to directly use purified mitochondria in patients should be considered carefully within a

specific context. When mitochondrial transfer is applied for tissue regeneration in vivo, several questions warrant future investigation, including whether mitochondria enter tissue-resident stem cells as well as how mitochondria influence downstream signalling pathways and stem cell fate.

Abbreviations

AAP	L-ascorbic acid 2-phosphate
ADSCs	Adipose-derived mesenchymal stem cells
AMPK	Adenosine 5′-monophosphate–activated protein kinase
ANAs	Acellular nerve allografts
Ara-C	Cytarabine
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BMMSCs	Bone marrow mesenchymal stem cells
BrdU +	5-bromo-2′-deoxyuridine
CCND1	cyclin D1
C-MYC	V-myc avian myelocytomatosis viral oncogene homolog
Cx43	connexin 43
DCX +	Doublecortin
DPSCs	Dental pulp stem cells
ECMO	Extracorporeal membrane oxygenation
EGFR	Epidermal growth factor receptor
EPCs	Endothelial progenitor cells
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
GATA-4	GATA binding protein 4
GFAP	Glial fibrillary acid protein
GJCs	Gap junctional channels
H_2O_2	Hydrogen peroxide
HGF	Hepatocyte growth factor
Hif-1α	Hypoxia-inducible factor1α
HO-1	Heme oxygenase-1
HSCs	Hematopoietic stem cells
HSPCs	Hematopoietic stem and progenitor cells
hUCMSCs	Human umbilical cord-derived mesenchymal stem cells
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
ICSI	Intracytoplasmic sperm injection
IONPs	Iron oxide nanoparticles
iPSCs	Induced pluripotent stem cells
IVF	In vitro fertilization
LPS	Lipopolysaccharide
MDS	Myelodysplastic syndromes
MDVs	Mitochondria-derived vesicles
MMP	Mitochondrial membrane potential
MSCs	Mesenchymal stem cells
mtDNA	Mitochondrial DNA
MTX	Methotrexate
NAC	N-acetyl-L-cysteine
NAD^+/NADH	Oxidized/reduced form of nicotinamide-adenine dinucleotide
NSCs	Neural stem cells
OGD	Oxygen-glucose deprivation
OXPHOS	Oxidative phosphorylation
PBMCs	Peripheral blood mononuclear cells
PDLSCs	Periodontal ligament stem cells
PI3K	Phosphoinositide 3-kinase
PINK1	PTEN-induced kinase 1
ROS	Reactive oxygen species
SCF	Stem cell factor
SCI	Spinal cord injury
SCs	Schwann cells
SHED	Stem cells from human exfoliated deciduous teeth
STAT3	Signal transducer and activator of transcription 3
T-ALL	T cell acute lymphoblastic leukemia
TEM	Transmission electron microscope
Th17	T helper 17 cells
Treg Cells	T regulatory cells
TNTs	Tunneling nanotubes
TOM	Translocase of the outer membrane
USCs	Urine derived stem cells
VE-cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells
WoSCC	Web of Science Core Collection

Acknowledgements
Not applicable.

Author contributions
Y.L., W.L.D. & C.Y. designed and outlined the review. Y.L. drafted the manuscript. Y.L. contributed to the material collection and analysis. Y.L. prepared the figures. W.L.D. & C.Y. critically reviewed and revised the manuscript. All authors approved the submission of the final version of the manuscript.

Funding
This work was supported by the Seed Fund for Basic Research (2302101829), the University of Hong Kong.

Data availability
No data was used for the research described in the article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Received: 28 November 2024 / Accepted: 8 April 2025
Published online: 21 May 2025

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