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A Guide for Studying Mitochondria Transfer

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Abstract

Mitochondria can shuttle between adjacent cells or travel to distant organs by breaking away from the parent cell and entering circulation. Here, we briefly review the state of the mitochondria transfer field and discuss a methodological framework for studying mitochondria transfer.

It has been almost 20 years since the discovery that organelles can be shuttled between cells in proximity via tube-like structures called tunneling nanotubes (TNTs)[1]. The first report of mitochondria transfer was suggested to occur through TNTs, although the mechanism was not fully elucidated[2]. Mitochondria can be transferred through cell contact-dependent processes such as TNTs, transient cellular fusion, or gap junction internalization (Figure 1)[3]. The latter is a process by which connexin-mediated gap junctions connecting two cells are internalized by one cell, resulting in engulfment of cellular material from the second cell[3]. Furthermore, mitochondria can also be transferred through cell contact-independent processes, where mitochondria are shed into the extracellular space and then taken up by other cells within the tissue or enter the circulation for delivery to cells in distant organs[3,4]. This comment focuses on the methodological considerations for the rigorous study of mitochondria transfer.

Types of Mitochondria Transfer

Cell contact-dependent mechanisms of mitochondria transfer involve separating intact mitochondria from the parent mitochondrial network and delivering them to the recipient cell. In contrast, transfer mechanisms involving extracellular mitochondria are more heterogenous. Intact mitochondria and/or mitochondria-derived vesicles (MDVs) can be released from cells either as naked mitochondria/MDVs or enclosed in a membrane bilayer derived from the endolysosomal system or plasma membrane. MDVs are budding

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structures that form on the larger mitochondrial network in response to oxidative stress. The budding vesicles contain oxidized mitochondrial components, which are pinched off and trafficked to the lysosome for degradation[5]. MDVs can be released from the cell as mitovesicles or enclosed in a lipid bilayer instead of being degraded[4,6–8]. Like whole mitochondria, some MDVs can respire and produce ATP[4] but some are devoid of either the mitochondrial matrix or outer membrane[5]. All forms of cell-free mitochondria can be characterized as extracellular vesicles (EVs), being a lipid bilayer-bound vesicle derived from and completely separated from the parent cell. However, there is no established nomenclature for distinguishing between these populations of extracellular mitochondria. For the sake of clarity, we refer to intact mitochondria or MDVs that are not enclosed in a membrane as “free mitochondria” and those surrounded by an additional lipid bilayer as “mitoEVs” (Figure 1). Further work is required to characterize the unique features of these subpopulations and provide a comprehensive, standardized nomenclature.

Functions of Mitochondria Transfer

A number of physiologic functions have been linked to mitochondria transfer and are reviewed in more detail elsewhere.[9] Two important functions of mitochondria transfer are to 1) improve the bioenergetics and function of the receiving cell and 2) outsource mitophagy to tissue-resident macrophages or astrocytes.[9] The first category necessitates the transfer of functionally competent mitochondria, whereas the second category involves the transfer of damaged mitochondria. Most of the mitochondria transfer studies report outcomes in category 1: for example, mesenchymal stem cells (MSCs) or astrocytes are capable of transferring mitochondria to various cells that were injured or depleted of mitochondrial DNA (mtDNA), thereby restoring aerobic respiration in the receiving cell and improving cell viability[2,10–12]. Healthy cell-free mitochondria can rescue respiratory deficits in macrophages with genetic defects in complex I activity, indicating that mitochondria transfer can rescue cell-intrinsic defects in mitochondrial metabolism[13]. It is also possible that mitoEVs play roles in the extracellular space as they have enzymatic activities and can respire, but this requires further examination[8].

Fewer studies demonstrated outcomes of mitochondria transfer in category 2. Trans-cellular mitophagy is important in several organs[4,6,14] and benefits the donor cell by preventing the accumulation of damaged mitochondria. In brown adipose tissue, the heart, and the brain, damaged cell-free mitochondria are taken up by macrophages for degradation, although other effects on macrophage function are possible[6,13–15].

It is important to highlight the value of understanding the mechanisms of mitochondria transfer, which provides key insights into potential functional outcomes. If mitochondria are transferred as mitoEVs, this may indicate that there will be broad functional changes in the receiving cell as EVs can carry many macromolecules including miRNA, mRNA, signaling proteins, and lipids because of their biosynthetic route[16]. If free mitochondria are transferred, the signals they carry to the target cell are likely restricted to mitochondrial signals such as reactive oxygen species (ROS), metabolites, and ATP. If the mechanism of transfer involves any form of cell-free mitochondria, the effect may be local or systemic. This contrasts with cell contact-dependent mechanisms, such as TNTs, where trafficking

of mitochondria occurs between nearby cells and likely requires intricate coordination of signaling between the participating cells within the tissue microenvironment.

Methodological Considerations for Demonstrating Mitochondria Transfer

Limitations

Several methods have been used to track the intercellular movement of mitochondria: tracking mitochondria stained with mitochondrial dyes, following sequence variants in mitochondrial DNA (mtDNA), or tracking mitochondria labeled with genetically encoded fluorescent proteins or tags. One limitation of all methods is that they cannot always distinguish between the transfer of intact mitochondria and the uptake of debris from dying cells. Therefore, evidence of live cells participating in the transfer should be acquired. However, apoptotic bodies can contain mitochondria[17] and mitochondria transfer detected from dying cells should be considered but distinguished from artifacts due to treatment-induced cell death. A second limitation is that fragments of mitochondria, such as MDVs, may not contain the molecule used to track mitochondria transfer. Therefore, care should be used in the interpretation of mitochondria transfer data.

Mitochondrial dyes

The use of mitochondrial dyes is common to show transfer of mitochondria *in vitro* in co-culture experiments, Transwell assays, conditioned media assays, or *in vivo* by adoptive transfer of stained cells into a host animal. Detection of dye transfer is generally done by fluorescence microscopy or flow cytometry. The use of membrane potential-dependent dyes provides evidence that the mitochondria being transferred can maintain a proton gradient, whereas membrane potential-independent dyes can track transfer of severely dysfunctional mitochondria. The major caveat to using these dyes is that they leak. Even if the excess dye is fully washed from the donor cell, the binding and release of the probe is in equilibrium, which results in portion of the dye leaking back out across the plasma membrane to stain other cells[18], producing false positive results. Similarly, the dye in transferred mitochondria may leak and directly stain nearby mitochondria in the receiving cells that were not transferred. Therefore, we suggest that mitochondrial dyes only be used as confirmatory evidence of mitochondria transfer that was already demonstrated by less problematic approaches and/or to characterize features of the transferred mitochondria such as membrane potential or relative ROS production.

Quantifying mtDNA

Quantifying mtDNA is a robust approach for tracking mitochondria but requires mtDNA sequence differences between the donating and accepting cell that can be detected by qPCR or sequencing. mtDNA variants could be species- or strain-specific[4,19]. mtDNA-based methods cannot always distinguish between transfer of mitochondria and free mtDNA. One way to mitigate this problem is treating donor mitochondria with DNase to ensure only protected mtDNA is transferred via intact mitochondria, if possible and practical. In addition, not all transferred mitochondrial material contains mtDNA, therefore, some mitochondria transfer events may not be detected. Lastly, mtDNA-tracking approaches may

not be readily conducive to detecting endogenous mitochondria transfer *in vivo*, unless there is a known difference in mtDNA variants between the donor and recipient cells.

Mitochondria reporter systems

Arguably, the best method for both *in vitro* and *in vivo* studies of mitochondria transfer is using a stable transgene encoding a mitochondrially localized tag or fluorescent protein[4,6,10,11,15,20,21]. This mitochondria reporter system can be either conditionally expressed to generate lineage-specific mitochondria reporter mice, expressed in all cells as a source of cells with labelled mitochondria or transduced into cells for ectopic expression *in vitro*. The presence of labeled mitochondria in a recipient cell is generally detected using immunofluorescence microscopy, flow cytometry, or western blotting. Appropriate controls are required. In the case of lineage-specific mitochondria reporter mice, it is important to demonstrate strict cell type-specific transgene expression, especially if using a Cre recombinase-based system, which can display a low-level leak of Cre expression in unintended cell types[22]. This can be detected because Cre-mediated recombination events will label *all* mitochondria in that cell, whereas transferred mitochondria will represent only a portion of the mitochondrial content of a recipient cell. It is useful to consider whether the chosen fluorescent protein or tag causes oxidative stress, mitochondrial dysfunction, or immunogenicity. Cell type-specific mitochondria reporter mice have been used successfully *in vivo* to demonstrate transfer of endogenous mitochondria between cells in adipose tissue, into circulation, and to distant organs[4,13]. However, this method is not particularly sensitive if using a dim fluorescent reporter protein, so low levels of *in vivo* mitochondria transfer may be challenging to detect. This drawback can be compounded if the target cell rapidly degrades incoming mitochondria, as this process would eliminate the mitochondrial signal. Short-term chloroquine treatment in mice or cells can diminish lysosomal function in the recipient cell to better visualize the transfer of extracellular mitochondria[4]. However, inhibiting lysosomal function promotes the release of mitochondria, which should be taken into consideration when interpreting the data[23].

When examining mitochondria transfer, it is essential to consider the methodological caveats described above and use more than one approach to detect mitochondria transfer. Use of microscopy is ideal if possible to verify internalization of the transferred mitochondrial material.

Guidelines for Assessing the Mechanism of Mitochondria Transfer

A first step is to determine if cell contact is required using co-cultures in Transwell systems or conditioned media experiments. If the mechanism of transfer does not require cell contact, it can occur through extracellular mitochondria. The presence of mitochondria should be confirmed in isolates from conditioned media or biofluids using standard methods of EV isolation[16]. Mitochondrial proteins can be quantified by western blot, Elisa, or flow cytometry. In addition, the presence of DNase-protected mtDNA and membrane potential can be determined. Unbiased strategies like proteomics and metabolomics can identify mitochondrial biomass in a sample[6]. To demonstrate the presence of fully assembled and functional mitochondria, the measurement of oxygen consumption rates, ATP production,

and/or activities of mitochondrial enzymes should be considered. Importantly, some populations of MDVs may not contain mtDNA, yet still maintain their membrane potential. Lastly, a way to identify if the purified extracellular material is free mitochondria or mitoEVs is to determine if mitochondrial components can be immunoprecipitated, purified, or stained with an antibody to an outer mitochondrial membrane (e.g., TOM20) and/or an EV membrane protein (e.g., CD63).

If the mechanism of mitochondria transfer requires cell-cell contact, it is likely occurring through TNTs or gap junction internalization, although other mechanisms like cellular fusion have been reported[3]. Mitochondria-containing TNTs can be visualized and quantified by confocal microscopy with fluorophore-labeled mitochondria combined with either an F-actin stain, plasma membrane stain, bright field microscopy, or electron microscopy to visualize TNTs[20,21]. TNTs can be disrupted chemically by inhibiting actin polymerization to implicate a role in mitochondrial transfer[24]. However, actin polymerization is required for cytoskeletal rearrangements and to produce subtypes of EVs, which might contain mitochondria. If TNTs cannot be detected, the role of gap junctions in mitochondria transfer can be determined via genetic manipulation of connexin expression.

Outlook

As this field matures, we are tasked with developing tools to manipulate the ability of cells to transfer mitochondria and robustly track mitochondria *in vivo*. As most mitochondria transfer studies have been conducted in cells or mice, there is no definitive evidence of mitochondria transfer in humans, which is hindering the deployment of therapeutic strategies based on mitochondria transfer. However, it is interesting to note that healthy human blood contains significant numbers of mitochondria, suggesting a role in the maintenance of healthy physiology[4,25]. Therefore, further work is required to establish the relevance of mitochondria transfer in human health and disease, and if enough healthy mitochondria can practically be administered to humans to influence disease progression.

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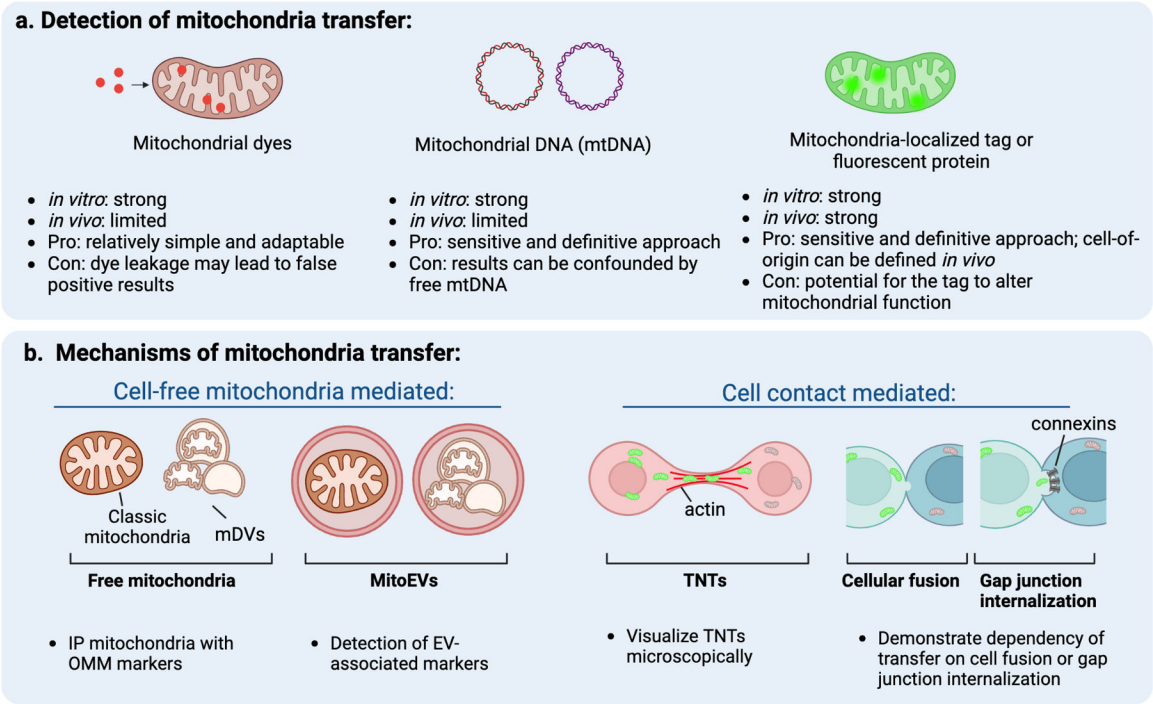

Figure 1. Methodologies for establishing mitochondria transfer mechanisms.

(a) Common methodologies used to detect mitochondrial transfer and **(b)** mechanisms of mitochondria transfer and experimental suggestions for distinguishing between them. MDVs; mitochondrial derived vesicles, IP; immunoprecipitation, OMM; outer mitochondrial membrane, TNTs; tunneling nanotubes. Images were created using biorender.com.