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A guide to studying mitochondria transfer

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

It has been almost 20 years since the discovery that organelles can be shuttled between nearby cells through tube-like structures called tunnelling nanotubes (TNTs)[1]. The first report of mitochondria transfer suggested that this can likewise occur through TNTs, although the mechanism was not fully elucidated[2]. Mitochondria can be transferred through cell-contact-dependent processes such as TNTs, through transient cellular fusion, or through internalization of gap junctions[3] (Fig. 1). The last is a process by which connexin-mediated gap junctions that connect two cells are internalized by one cell, which results in engulfment of cellular material from the second cell[3]. Furthermore, mitochondria can be transferred through cell-contact-independent processes, whereby mitochondria are shed into the extracellular space and then either are taken up by other cells within the same tissue or enter the circulation for delivery to cells in distant organs[3,4]. This Comment focuses on 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. By contrast, transfer mechanisms that involve extracellular mitochondria are more heterogenous. Intact mitochondria and/or mitochondria-derived vesicles (MDVs) can be released from cells, either as naked mitochondria or MDVs, or enclosed in a membrane bilayer derived from the endolysosomal system or plasma membrane. MDVs are budding 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 the outer membrane[5]. All forms of cell-free mitochondria can be characterized as extracellular vesicles (EVs), being lipid bilayer-bound vesicles that are derived from, but 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 refer to those surrounded by an additional lipid bilayer as 'mitoEVs' (Fig. 1). Further work is needed to characterize the unique features of these subpopulations and to provide a comprehensive, standardized nomenclature.

Functions of mitochondria transfer

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

Fewer studies have demonstrated outcomes of mitochondria transfer in the second category. Transcellular mitophagy is important in several organs[4,6,14] and benefits the donor cells 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 also 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: EVs can carry many macromolecules, including microRNAs, messenger RNAs, signalling proteins and lipids, because of their biosynthetic route[16]. If free mitochondria are transferred, the signals that they carry to the target cell are likely to be restricted to mitochondrial signals, such as reactive oxygen species, 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 — in which trafficking of mitochondria occurs between nearby cells and probably requires intricate coordination of signalling between the participating cells within the tissue microenvironment.

Methodological considerations for demonstrating mitochondria transfer

Limitations. Several tools have been used to track the intercellular movement of mitochondria, including mitochondrial dyes, sequence variants in mtDNA and genetically encoded fluorescent proteins or

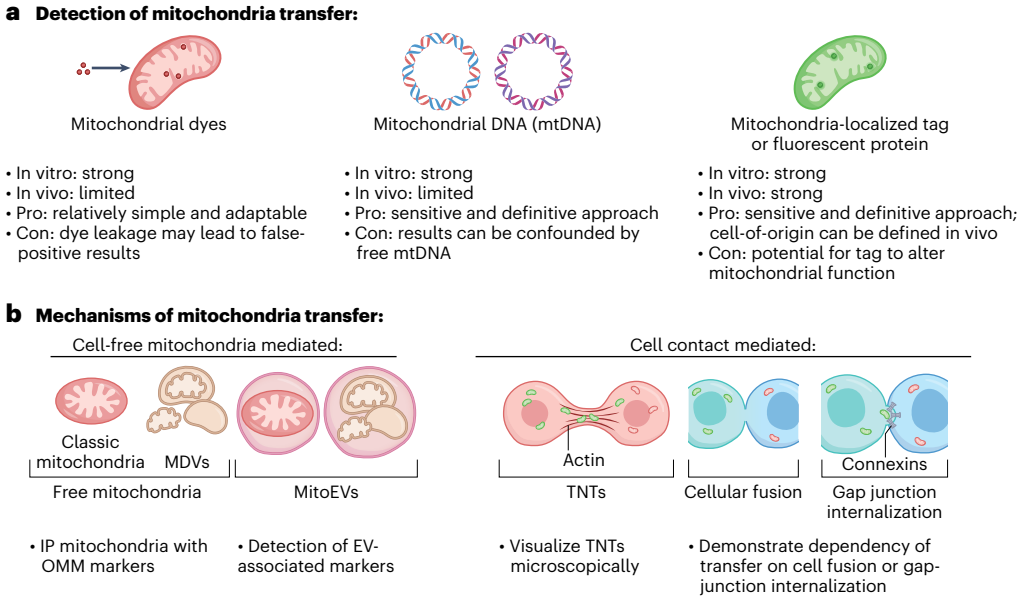

Fig. 1 | Methodologies for establishing mitochondria transfer mechanisms. a, Common methodologies used to detect mitochondria transfer. **b**, Mechanisms of mitochondria transfer and experimental suggestions for distinguishing between them. IP, immunoprecipitation; OMM, outer mitochondrial membrane. Created with BioRender.com.

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 obtained. However, apoptotic bodies can contain mitochondria[17], and mitochondria transfer detected from dying cells should be considered but distinguished from artefacts due to treatment-induced cell death. A second limitation is that fragments of mitochondria, such as MDVs, may not contain the molecule chosen to track mitochondria transfer (i.e., mtDNA) and thus will give a false negative result. Therefore, care should be taken when interpreting data on mitochondria transfer.

Mitochondrial dyes. The use of mitochondrial dyes is common to show transfer of mitochondria in vitro in co-culture experiments, Transwell assays or 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 dyes that depend on membrane potential provides evidence that the mitochondria being transferred can maintain a proton gradient, whereas dyes that are independent of membrane potential can track the transfer of severely dysfunctional mitochondria.

The main caveat in the use of 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 a 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 be used only 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 production of reactive oxygen species.

Quantifying mtDNA. Quantifying mtDNA is a robust approach for tracking mitochondria, but it requires differences in mtDNA sequence between the donating and accepting cells that can be detected by quantitative PCR or sequencing. mtDNA variants could be species or strain specific[4,19]. mtDNA-based methods cannot always distinguish between the transfer of mitochondria and free mtDNA. One way to mitigate this problem is to treat donor mitochondria with DNase in order to ensure that 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. Finally, mtDNA-tracking approaches may not be readily conducive to the detection of endogenous mitochondria transfer in vivo, unless there is a known difference between donor cells and recipient cells in their mtDNA variants.

Mitochondria reporter systems. Arguably the best method for both in vitro and in vivo studies of mitochondria transfer is the use of a stable transgene that encodes a mitochondrially localized tag or fluorescent protein[4,6,10,11,15,20,21]. This mitochondria reporter system can be 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 labelled mitochondria in a recipient cell is generally detected by 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 a system based on Cre recombinase is used, as such systems 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 to demonstrate in vivo transfer of endogenous mitochondria between cells in adipose tissue, into the circulation and to distant organs[4,13]. However, this method is not particularly sensitive if a dim fluorescent reporter protein is used, 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 would eliminate the mitochondrial signal. Short-term treatment of mice or cells with chloroquine can diminish lysosomal function in the recipient cell for better visualization of 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 to 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 mechanisms of mitochondria transfer

A first step is to determine whether 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 through the use of 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 such as 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. Notably, some populations of MDVs may not contain mtDNA, yet still maintain their membrane potential. Finally, a way to identify whether the purified extracellular material comprises free mitochondria or mitoEVs is to determine whether mitochondrial components can be immunoprecipitated, purified or stained with an antibody to an outer mitochondrial membrane protein (such as TOM20) and/or an EV membrane protein (such as CD63).

If the mechanism of mitochondria transfer requires cell-to-cell contact, it is likely to be occurring through TNTs or internalization of gap junctions, although other mechanisms such as cellular fusion have been reported[3]. Mitochondria-containing TNTs can be visualized and quantified by confocal microscopy with fluorophore-labelled mitochondria combined with either an F-actin stain, a plasma membrane stain, brightfield microscopy or electron microscopy for visualization of TNTs[20,21]. TNTs can be disrupted chemically through inhibition of actin polymerization to delineate 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, researchers are tasked with developing tools to manipulate the ability of cells to transfer mitochondria and robustly track mitochondria in vivo. As most studies of mitochondria transfer have been carried out in cells or mice, there is no definitive evidence for the process in humans, which hinders the deployment of therapeutic strategies based on mitochondria transfer. However, it is interesting that healthy human blood contains substantial numbers of mitochondria, suggestive of a role for mitochondria in the maintenance of healthy physiology[4,25]. Therefore, further work is needed to establish the relevance of mitochondria transfer in human health and disease, and to determine whether enough healthy mitochondria can practically be administered to humans to influence disease progression.

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Competing interests

J.R.B. has pending and issued patents related to mitochondria transfer and obesity; has been a consultant for DeciBio and Flagship Pioneering within the past 12 months; receives royalties from Springer Nature; and is on the Scientific Advisory Board for LUCA Science.

Additional information

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