Bioenergetic crosstalk between mesenchymal stem cells and various ocular cells through the intercellular trafficking of mitochondria

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Running title: Trait of mitochondrial transfer from MSCs to ocular cells
Methods

Transwell assay

We conducted Transwell assay to evaluate the necessity of physical contact for intercellular mitochondrial transfer. CellTracker red-labeled CECs (CellTracker™ Red CMTPX Dye, Invitrogen, C34552, USA) on the lower chamber of Transwell were co-cultured with 1) normal medium, 2) supernatant of MSCs, 3) MSCs on the upper chamber of Transwell, and 4) MSC on the lower chamber of Transwell respectively. After 24 h co-cultivation, the red-CECs were harvested for FACS sorting and images were taken using the laser scanning confocal microscope.

Flow Cytometry

Mitochondrial recipient cells were prepared for OCR, transcriptome and ending fate of the internalized mitochondria analysis. Briefly, CellTrace violet-labeled cells and Mito-COX8-GFP-labeled MSCs were co-cultured at a ratio of 1:1 in a 6-well plate for 24 h. The positive beads were gated on the FL1-FITC, FL6-Pacific blue plot. Pacific blue and FITC specific fluorescence were assessed.

Immunofluorescence and immunocytochemistry analyses

To obtain images of live cells, we transfected mitochondrial donor cell with Mito-COX8-GFP virus. Before co-culture, we labeled the mitochondrial recipient cells with CellTrace violet or CellTracker red. After fixation, cells were permeated for 5 min with 0.1% Triton X-100, followed by 30 min cultivation with 1% bovine serum albumin. Next, we performed staining of the cells with Alexa Fluor™ 647 phalloidin for 20 min at room temperature. After two-fold washing with PBS, the cells were
air-dried and mounted in a permanent mounting medium.

**Evaluation of mitochondrial transfer in whole mount retina**

At day 3 after subretinal injection, eyes of mice were enucleated and fixed in 4% PAF (paraformaldehyde) for 30 min at 4 °C followed by a cornea puncture and an extra of 30 min’ fixation. Then, the retina was detached carefully from the underlying RPE and sclera. After soaking with 5% BSA and 0.5% Triton-X-100, the retinas were incubated with anti-Opsin antibody (Millipore Cat# AB5405, RRID: AB_177456) at 4 °C overnight followed by secondary antibody and DAPI at room temperature for 2 h. After washing with PBST for 3 times, retina was flatted and mounted. Fluorescent images were taken using the fluorescent microscope.
**Figure and video legends**

**Figure S1.** Cell labeling.  
A. lentiviral-mediated mitochondrial-specific fragment fused with green florescence protein (Mito-COX8-GFP) was used to label mitochondria of donors.  
B. MSCs labeled with Mito-COX8-GFP.  
C. CECs labeled with Mito-COX8-GFP.  
D. CECs labeled with CellTrace violet. Scale bars: 75 μm.

**Figure S2.** Transwell assay to evaluate the necessity of physical contact for cell-cell mitochondrial transfer.  
A-D. CellTracker red-labeled CECs on the lower chamber of Transwell were co-cultured with A) normal medium, B) supernatant of MSCs, C) MSCs on the upper chamber of Transwell, D) and MSC with physical contact on the lower chamber of Transwell respectively. Mito-COX8-GFP signals were detected in the cytoplasm of the red-CECs in D (arrowhead). Arrow Scale bars: 20 μm.

**Figure S3.**  
A. Co-culture of GFP-MSCs and violet-rot-CECs; (A’) magnified image of box-A’. Arrow show MSC-derived mitochondria in recipient CECs.  
B. Co-culture of cytochalasin B (CB) pretreated GFP-MSCs and violet-rot-CECs; (B’) magnified image of box-B’.  
C. Intercellular mitochondrial transfer rate.  
D. Bar graph shows gene expression of ACTN1, NEXN and CAP2 in rot-CEC (MSC) (fold change of rot-CEC (MSC) vs. rot-CEC). GFP-MSC\(^{CB}\): GFP-MSC pretreated with 350 nM CB for 24 h before coculture; rot: rotenone; rot-CEC\(^{CB}\): rot-CEC pretreated with 350 nM CB for 24 h before coculture.

**Figure S4.**  
A. Top 20 pathway enrichment terms for differentially expressed intersection mRNAs, including up-regulated pathway and down-regulated pathway
(rot-CECs v.s. rot-CECs (MSC)). **B.** Hierarchical clustering of CEC signature genes. Data are presented as fragments per kilobase of transcript per million mapped reads (FPKM). Blue: downregulated expression; Red: upregulated expression; rot: rotenone.

**Figure S5.** Proliferation potential of CECs. Proliferative capacity of FACS-sorted CECs from different groups was evaluated, including CECs, rot-CECs, CECs with MSC supernatant, rot-CECs with MSC supernatant and rot-CECs cocultured with MSC. One-way ANOVA, mean ± SD, ns: no significant difference, *P < 0.05*, n = 4.

**Figure S6.** Transferred mitochondria and the host lysosomes in the recipient cells. The distribution of host lysosomes (Fuchsia) and MSC-derived mitochondria (Green) in the recipient cells at 48 h after co-cultivation.

**Video S1.** A video of reconstructed Z-stack images shows MITO-COX8-GFP-tagged mitochondria within opsin positive cone.

**Video S2.** Camera phase of a Time-Lapse video shows that mitochondria were being transferred from MSCs to CECs.

**Video S3.** A video of reconstructed Z-stack images shows GFP-MSCs were co-cultured with violet-CECs and GFP-mitochondria were transferring from MSC to CEC via a tunneling nanotubes (TNTs) structure.

**Video S4.** Camera phase of a Time-Lapse video shows the distribution and movement of transferred mitochondria and host lysosomes within violet-CECs.

**Video S5.** Camera phase of a Time-Lapse video shows the GFP-mitochondria (from
MSCs) and host lysosomes were transferring from one 661w to another.

**Video S6.** Camera phase of a Time-Lapse video shows the GFP-mitochondria were gradually packaged in 3-5 μm round bubbles and excreted to extracellular vesicles.

**Supplemental Figures**

**Figure S1**

**Figure S2**
Figure S3

A
rot-CEC + GFP-MSC
Violet-CEC
GFP-Mitochondria
F-actin

A'

B
rot-CEC\textsuperscript{cb} + GFP-MSC\textsuperscript{cb}
Violet-CEC
GFP-Mitochondria
F-actin

B'

C
Transfer Rate %
rot-CEC
GFP-MSC
rot-CEC\textsuperscript{cb}
GFP-MSC\textsuperscript{cb}

D
Gene expression
ACTN1
NEXN
CAP2

Fold Change
0
2
4
6
Figure S5

![Bar graph showing cell proliferation percentages with error bars.](image)

Figure S6

![Images showing different cellular structures labeled with different colors and scales.](image)