nature portfolio

Peer Review File

Super mitochondria-enriched extracellular vesicles enable enhanced mitochondria transfer

Corresponding Author: Professor Hu-Lin Jiang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors show that CAP/CD38 complex stimulation leads to increased release of extracellular mitochondria from MSCs in vitro. These ex-Mito are about 600 nm in diameter and co-express tetraspannins, though it is not clear what proportion are in EVs. These “super-MEVs” boost mitochondrial metabolism of GM10742 cells. Next, the used an LHON mouse model and a Rot-induced mouse model and show that the “Super-MEVS” ameliorate eye disease in both. This is an interesting manuscript that builds on 2 recent studies showing that mitochondria transplants can ameliorate mitochondrial diseases and extends the disease indications to LHON. The conceptual advance from the CD38-induced “Super-MEVs” is unclear and needs to be studied more deeply to understand mechanism.

Major concerns:
1. The authors imply that all of the released mitochondria are in EVs but this is not demonstrated. The percent of particles in EVs must be quantified.
2. Fig 3 and Fig 6 utilize mitochondrial dyes to track uptake. These are very leaky and create false positive results. They should not be used without very careful controls and verification with other methods. In general, mitochondrial dyes are strongly discouraged for the purpose of studying mitochondria transfer.
3. The term MEVs is inconsistent with a recent consensus statement by the International Committee of Mitochondria Transfer and Transplantation published in Nature Metabolism earlier this year.
4. The mechanism(s) by which super-MEV mito gain access to the cytoplasm must be defined.
5. How does CD38 signaling promote release of ex-Mito?
6. Does daratumimab prevent or enhance release of “super-MEVs” or other extracellular mito from MSCs?
7. Figure 5 is just a cartoon with no data.

Reviewer #2

(Remarks to the Author)
This manuscript written by Wang et al. reported a potential method to promote the generation of mitochondria-enriched extracellular vesicles (MEVs) by MSCs through a genetic modification. They further show the therapeutic potential of their MEVs via the strategy of mitochondrial transfer. The work is of certain significance. However, the reviewer still has several concerns about the results and conclusions, and a further revision is necessary.

1. The correlation between CD38 expression and MEV release has already been reported in previous studies (e.g. PMID: 36754021, 27466127). In this manuscript, authors applied this signal pathways to promote the production of MEVs via gene transfection using their previous reported gene vectors. Hence, the novelty of the present study in comparison with previous studies is suggested to further address.
2. The mechanisms by which intracellular Ca2+ regulates MEV production and how elevated Ca2+ levels promote MEV release are suggested to clarify.
3. As mentioned by the authors, the high quality of mitochondria is important. In this case, why the authors used MSCs as the donor cell of MEV, which usually the amount of mitochondria is limited ? Will it be more effective by using the donor cell with more active mitochondria, such as myocardial cells ?
4. It was previous reported that the package of impaired mitochondria in EVs is one of the approaches to eliminate the

dysfunctional mitochondria (PMID: 34418352; 37108168). Therefore, whether the promotion of MEV will lead to more damaged mitochondria in EVs or how the authors to avoid the package of impaired mitochondria in their MEV?
5. As introduced by the authors that the primary cause of LHON is the G11778A mitochondrial DNA mutation. Will the treatment using MEV repair this mitochondrial DNA mutation ? If not, what is the therapeutic mechanism underlying the mitochondrial transfer? Also, if the administration of MEV is terminated, will the symptom of LHON recovered. More studies to figure out this issue is suggested, especially the long-term therapeutic potentials after the MEV administration.
6. How about the stability of mitochondria in MEV?
7. The quality of the mitochondria in super-MEV and super-donor MSCs is suggested to compare.
8. The Ca^{2+} overload is often associated with mitochondrial damage, which could pose potential risks for MEV. While the study examined changes in mitochondrial membrane structure in CAP/CD38-modified MSCs and reported that elevated Ca^{2+} levels significantly suppressed mPTP opening compared to untreated MSCs, this observation appears inconsistent with existing literature suggesting that Ca^{2+} elevation promotes mPTP opening (PMID: 36410526, 31944918). Please discuss.
9. In Fig1k, why there is no difference between Lipo3000 and CAP regarding to the basal respiration but has difference in terms of maximum respiration?
10. More proofs are required to support the conclusion that the mitochondria in super-MEV are functional, by saying “higher content of functional mitochondria in Super-MEVs (Fig. 3A, B).” The current data can support more content of mitochondria, but hardly to say more functional mitochondria.
11. Similarly, in the evaluation of the protective effects of MEVs on mitochondria, the authors used MTG fluorescence intensity as an indicator of mitochondrial changes. However, this approach lacks characterization of mitochondrial functional alterations under different stress conditions. Additionally, in the stimulation experiments, the rationale for selecting the Ca^{2+} concentration gradient (0, 0.0001, 1.25, 2.5 mM) remains unclear and requires further clarification.
12. “SuperMEV treatment increased COX IV expression compared with Ctrl-MEV treatment (Fig. 3C and Fig. S12B)”. However, the data showed there is no statistic difference among Super-MEVs, Ctrl-MEV and Lipo-MEV. Partially, there are several data including the therapeutic outcomes showed no statistic difference between Super-MEVs and Lip-MEV. What are the major advantages of CAP, except the biocompatibility?
13. Will the regulation of CD38/IP3R/Ca^{2+} affect other functions of MSCs, such as the migration and differentiation?
14. Although several data demonstrated the statistic difference of Super MEVs in comparison with Ctril-MEVs or Lipo-MEVs, the increasing times of the values looks not so obviously. Please discuss it.
15. Fig.1c, the background of IP3R and GAPDH look so different. What is the reason?
16. Fig.5 may be more suitable listed in the end of Fig.6.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my concerns satisfactorily. Congratulations on a nice set of work.

Reviewer #2

(Remarks to the Author)
The revised version of the manuscript answered all the issues of the reviewer and the quality of this work has been improved notably. It is acceptable now.

Responses to the comments of the Reviewers

We would like to express our sincere gratitude to the reviewers for your time, effort, and constructive feedback throughout the review process. The reviewers' comments are presented in black and numbered, and our point-by-point responses are provided in blue. Corresponding revisions in the main manuscript and Supporting Information are also marked in blue for clarity.

Reviewer #1 (Remarks to the Author):

The authors show that CAP/CD38 complex stimulation leads to increased release of extracellular mitochondria from MSCs in vitro. These ex-Mito are about 600 nm in diameter and co-express tetraspannins, though it is not clear what proportion are in EVs. These "super-MEVs" boost mitochondrial metabolism of GM10742 cells. Next, the used an LHON mouse model and a Rot-induced mouse model and show that the "Super-MEVS" ameliorate eye disease in both. This is an interesting manuscript that builds on 2 recent studies showing that mitochondria transplants can ameliorate mitochondrial diseases and extends the disease indications to LHON. The conceptual advance from the CD38-induced "Super-MEVs" is unclear and needs to be studied more deeply to understand mechanism.

Response: Thank you for your kind words and acknowledgment of our work. We greatly appreciate your valuable comments. In response to your concerns, we conducted additional key experiments to explore the proportion in EVs and investigate the underlying mechanism of CD38-induced generation of Super-EV-Mito. We hope that these additional experiments and adjustments address your concerns adequately. Please find below our detailed responses to each of the comments you provided.

Major concerns:

1. The authors imply that all of the released mitochondria are in EVs but this is not demonstrated in EVs must be quantified.

Response: We sincerely appreciate the reviewer's valuable suggestion. As correctly pointed out, not all released mitochondria are encapsulated within EVs; a portion may indeed exist in the form of free mitochondria. In response to this concern, we conducted nano-flow cytometry analysis to quantify the proportion of released mitochondria encapsulated in EVs based on the previously published study[1]. The result showed that approximately 24.87 ± 2.76% of mitochondrial particles were double-positive, indicating their encapsulation within EVs, while the remaining mitochondria were FITC-positive, suggesting that they existed as free mitochondria (Fig. 2D).

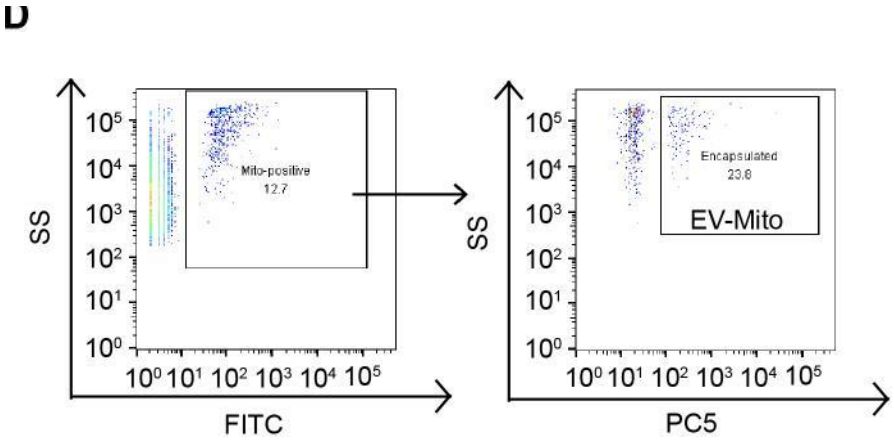

Fig. 2. Preparation and characterizations of Super-EV-Mito. (D) The proportion of released mitochondria encapsulated in Super-EV-Mito using nano-flow cytometry analysis. FITC: mEmerald-TOMM20 labeled mitochondria, PC5: CD81 in EVs.

However, the double-positive population in nano-flow cytometry only reflects the proportion of mitochondria associated with EVs and does not directly visualize how many mitochondria are present within individual EV. To address this, we further quantified the mitochondrial content within EVs using TEM analysis. Statistical evaluation of Super-EV-Mito TEM images revealed that, on average, 6 out of every 10 released mitochondria were encapsulated in EVs, while the remaining 4 existed in a free form (Fig. 2E and Fig. S8). These experimental results and the associated discussion have been added to the revised manuscript.

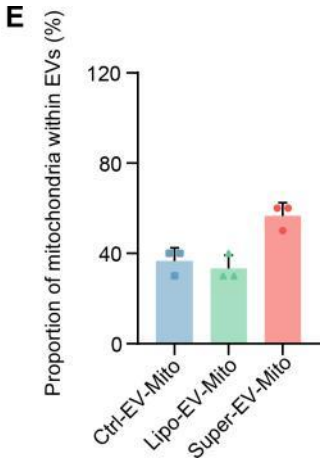

Fig. 2. Preparation and characterizations of Super-EV-Mito. (E) The proportion of mitochondria within EVs calculated by TEM images.

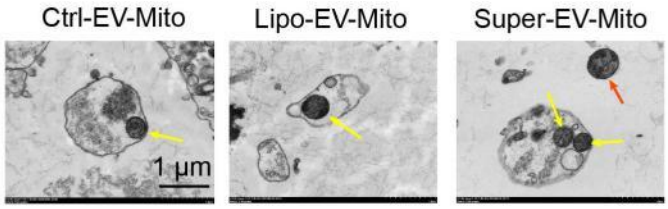

Fig. S8. Representative images of different EV-Mito group. Yellow arrows: released mitochondria enclosed in EVs; red arrows: free mitochondria not enclosed in EVs.

1. Hayakawa, K. et al. Transfer of mitochondria from astrocytes to neurons after stroke. *Nature* **535**, 551-555 (2016).

0. Fig 3 and Fig 6 utilize mitochondrial dyes to track uptake. These are very leaky and create false positive results. They should not be used without very careful controls and verification with other methods. In general, mitochondrial dyes are strongly discouraged for the purpose of studying mitochondria transfer.

Response: Thank you very much for the valuable comments. Following your suggestion, we transduced MSCs with a viral vector encoding mEmerald-TOMM20 to specifically and stably label mitochondria, thereby avoiding issues associated with dye leakage and reducing the risk of false-positive mitochondrial localization. We then utilized mEmerald-TOMM20-labeled Super-EV-Mito to track uptake in recipient cells as shown in Fig. 3A and Fig. 6A (Fig. 3A and

Fig. 5A in the revised manuscript). The results demonstrated that the mean fluorescence intensity (MFI) of mEmerald-TOMM20 in recipient cells treated with Super-EV-Mito was significantly higher than that in other groups, indicating that Super-EV-Mito facilitated the cellular uptake of exogenous mitochondria. We have incorporated these experiments and the corresponding discussion into the revised manuscript.

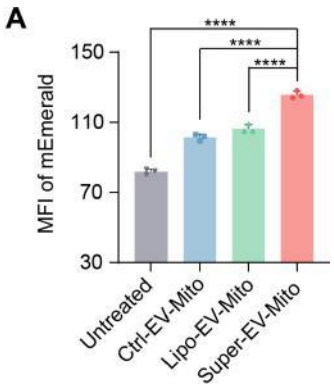

Fig. 3. Super-EV-Mito restore the mitochondrial functions of GM10742 cells. (A) Cell uptake of different groups evaluated by flow cytometry analysis.

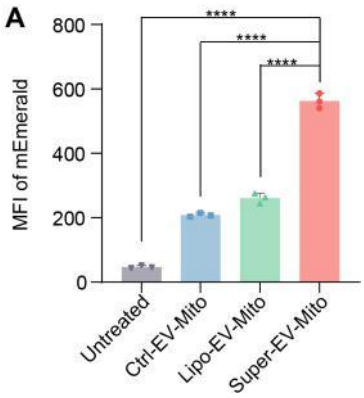

Fig. 5. Protection effect of the Super-EV-Mito in Rot-induced cell and animal model. (A) Cell uptake of mEmerald-TOMM20-labeled EV-Mito evaluated by flow cytometry analysis.

3. The term MEVs is inconsistent with a recent consensus statement by the International Committee of Mitochondria Transfer and Transplantation published in Nature Metabolism earlier this year.

Response: Thank you for your constructive suggestion. In accordance with your advice, we carefully reviewed the recent consensus statement by the International Committee of Mitochondria Transfer and Transplantation published in Nature Metabolism[1]. Based on this, we have revised the terminology in the manuscript, changing "MEV" to "EV-Mito" to align with the recommended nomenclature.

1. Brestoff, J.R. et al. Recommendations for mitochondria transfer and transplantation nomenclature and characterization. *Nat. Metab.* **7**, 53-67 (2025).

4. The mechanism(s) by which super-MEV mito gain access to the cytoplasm must be defined.

Response: We thank you very much for the valuable suggestion. According to your suggestion, we conducted cellular uptake inhibition experiments to investigate the mechanism by which Super EV-Mito were internalized into the cytoplasm of recipient cells. As shown in Fig. S14, we found that Super-EV-Mito enters recipient cells via multiple endocytic pathways, with macropinocytosis and caveolin-mediated endocytosis being the predominant mechanisms. These experiments and the corresponding discussion have been added to the revised manuscript.

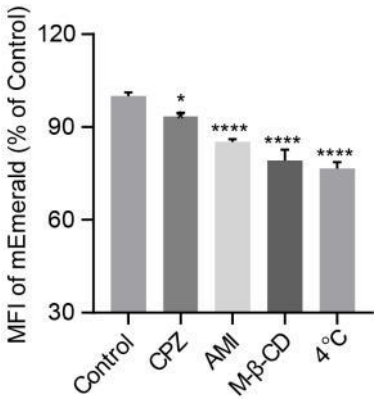

Fig. S14. The cellular uptake mechanism study of Super-EV-Mito. CPZ: chlorpromazine, AMI: amiloride; M-f3-CD: methyl-f3-cyclodextrin. *n* = 3. Statistical significances were performed via one-way ANOVA with Tukey's HSD post hoc test. All significance comparisons were conducted relative to the control group.

5. How does CD38 signaling promote release of ex-Mito?

Response: Thank you very much for the valuable comment. The mechanism by which CD38 signaling promotes the release of EV-Mito involves several key steps (Fig. 1M):

1. CD38 catalyzes the synthesis of the calcium-mobilizing second messenger cyclic ADP-ribose (cADPR), resulting in a significant elevation of cytoplasmic calcium levels (Fig. S7A).
2. Mitochondria are major intracellular calcium stores[1,2]. We observed that upregulation of CD38 expression led to increased expression of IP3 receptors (IP3Rs) on the endoplasmic reticulum (ER), particularly those located at ER-mitochondria contact sites. These IP3Rs rapidly mediate Ca^{2}+ transfer to mitochondria, thereby stimulating mitochondrial oxidative metabolism.
3. Previous studies have demonstrated that elevated intracellular Ca^{2}+ levels can trigger the fusion of vesicles with the plasma membrane through a Synaptotagmin/SNARE complex-mediated exocytosis mechanism, thereby promoting the release of extracellular vesicles (EVs) and their cargo[3,4]. Specifically, Ca^{2}+ elevation activates the calcium sensor Synaptotagmin on vesicles, inducing a conformational change that facilitates the assembly and activation of the SNARE complex, ultimately accelerating the membrane fusion and exocytosis of EV-Mito.

In response to the reviewer's suggestion, we systematically investigated the mechanism by which CD38 signaling promotes ex-Mito release. First, transcriptomic and GO analyses comparing normal MSCs and CD38-overexpressing MSCs revealed that CD38 upregulation altered calcium-dependent pathways, including enrichment in extracellular region and space, positive regulation of cytosolic Ca^{2}+ concentration, and enhanced mitochondrial fission (Fig. 1A). These data supported the hypothesis that CD38 regulated both mitochondrial function and EV-Mito release through Ca^{2}+-dependent mechanisms. In addition, CD38 catalyzes the synthesis of the calcium-mobilizing second messenger cyclic ADP-ribose (cADPR), resulting in a significant elevation of cytoplasmic calcium levels (Fig. S7A).

Further investigation demonstrated that CD38 overexpression significantly upregulated IP3R expression (Fig. 1B, C and Fig. S7B). Given that IP3Rs located at ER-mitochondria contact sites mediate rapid calcium transfer, we measured mitochondrial calcium levels and found them significantly increased upon CD38 upregulation (Fig. 1D). Knockdown of IP3R expression via siRNA (IP3R KD) significantly inhibited CD38-induced IP3R activation (Fig. 1G) and reduced mitochondrial calcium accumulation (Fig. 1H), which in turn resulted in a

marked decrease in EV-Mito release (Fig. 1I).

To elucidate the mechanism by which elevated intracellular calcium promotes EV release, we first confirmed that CD38-mediated $Ca^{2}+$ upregulation significantly increased EV-Mito secretion (Fig. 1E). Pharmacological inhibition studies further demonstrated that the release of EV-Mito was markedly suppressed by actin polymerization inhibitors, whereas microtubule inhibitors had no discernible effect (Fig. S7C and D), suggesting that EV-Mito release is actin-dependent but independent of microtubule dynamics[5]. Previous studies have shown that intracellular $Ca^{2}+$ elevation activates the calcium sensor Synaptotagmin, a key regulator of $Ca^{2}+$-dependent exocytosis. Synaptotagmin contains two conserved C2 domains (C2A and C2B) that mediate $Ca^{2}+$-dependent interactions with phospholipids and SNARE proteins[6]. Upon $Ca^{2}+$ binding, these C2 domains undergo conformational changes that enable Synaptotagmin to interact with membrane lipids and facilitate the assembly of the SNARE complex, composed of proteins such as SNAP-25, syntaxin, and VAMP. The SNARE complex mediates the docking and fusion of vesicles with the plasma membrane, ultimately promoting EV release[7]. Taken together with our data, it could be speculated that CD38-induced $Ca^{2}+$ elevation enhances EV-Mito production through a Synaptotagmin/SNARE complex-mediated exocytosis mechanism, which was tightly regulated by actin polymerization. This mechanistic insight highlighted a calcium-sensitive, actin-dependent vesicle trafficking pathway as a key contributor to EV-Mito secretion.

These mechanistic insights and the corresponding experimental results have been added to the revised manuscript, and the relevant data have been reorganized and presented in the updated Fig. 1 and Fig. S7.

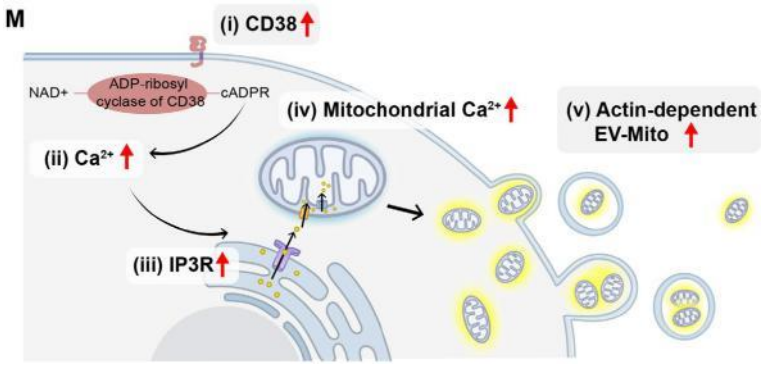

Fig. 1. CD38 signal increases mitochondrial calcium via IP3R and enhances extracellular mitochondria. (M) A schematic diagram illustrating the release of EV-Mito from MSCs regulated by the CD38/IP3R/Ca^{2+} pathway.

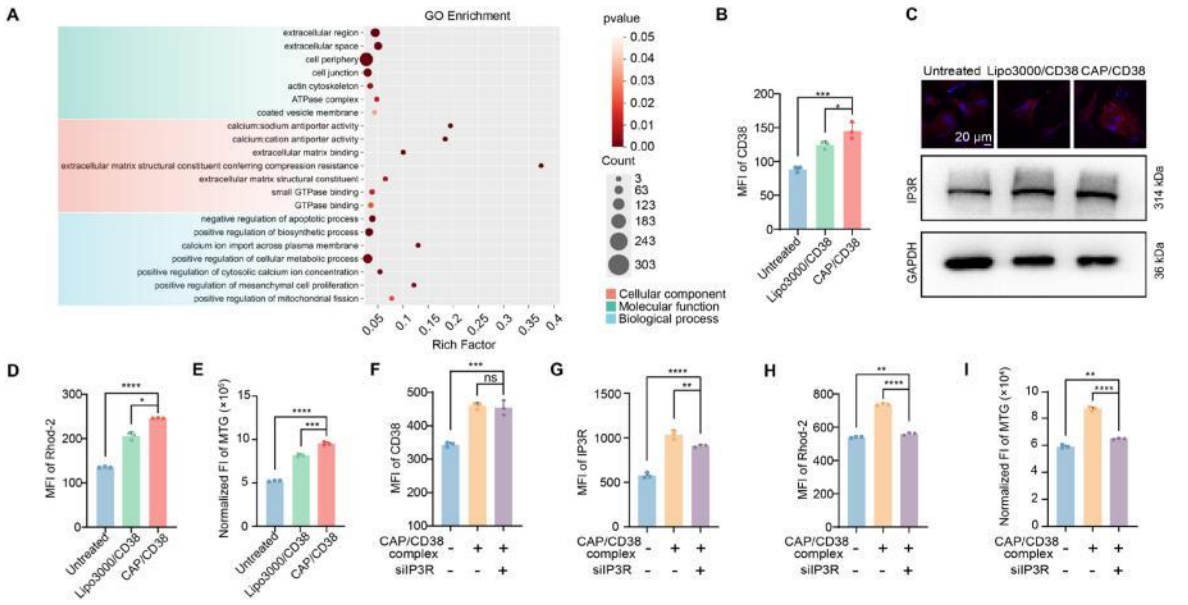

Fig. 1. CD38 signal increases mitochondrial calcium via IP3R and enhances extracellular mitochondria. (A) GO analysis based on the significant differentially expressed genes between untreated and CAP/CD38-treated MSCs. (B) Gene expression of CD38 in MSCs after different treatments. (C) Gene expression of IP3R in MSCs after different treatments. (D) The determination of mitochondrial Ca^{2+}. (E) Normalized fluorescence intensity of Mitotracker green (MTG) of extracellular mitochondria in a condition medium. (F-I) CD38 expression (F), IP3R expression (G), content of mitochondrial Ca^{2+} (H), and normalized fluorescence intensity of MTG (I) of extracellular mitochondria with and without IP3R knockdown.

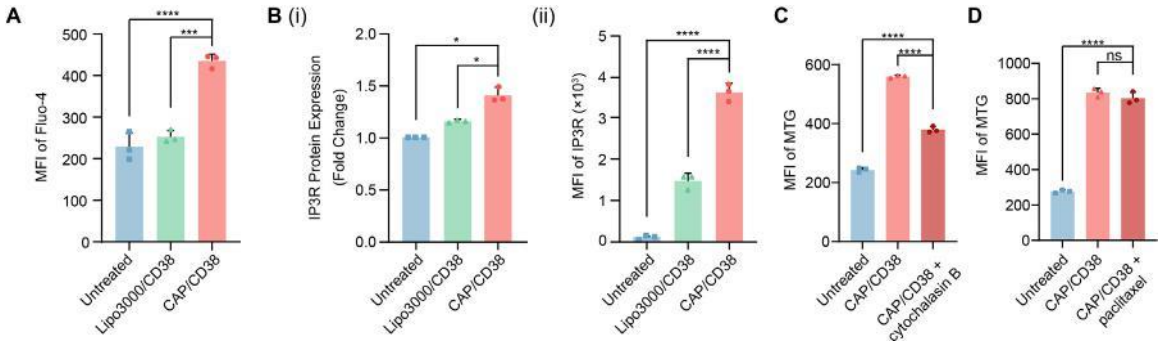

Fig. S7. IP3R protein expression changes in MSCs following CD38 transfection and the release of EV-Mito under different inhibitors. (A) Intracellular Ca^{2+} levels in MSCs after different treatment. (B) IP3R protein expression quantified by Western blot and immunofluorescence. (C) The release of EV-Mito with or without actin inhibitors (cytochalasin B). (D) The release of EV-Mito with or without tubulin inhibitor (paclitaxel). $n = 3$. Statistical significances were performed via one-way ANOVA with Tukey's HSD post hoc test (A, B (ii), C, D) or Games-Howell test (B (i)).

1. Wang, Y. et al. Enforced expression of mitochondrial calcium uniporter in donor T cells abolishes gvhd progression. *Blood* **144**, 3403 (2024).

2. Cartes-Saavedra, B., Ghosh, A. & Hajnoczky, G. The roles of mitochondria in global and local intracellular calcium signalling. *Nat. Rev. Mol. Cell Biol.* **26**, 456-475 (2025).
3. Kaeser, P. S. et al. RIM proteins tether Ca^{2+} channels to presynaptic active zones via a direct PDZ-domain interaction. *Cell* **144**, 282-295 (2011).
4. Chapman, E. R. Synaptotagmin: a Ca^{2+} sensor that triggers exocytosis? *Nat. Rev. Mol. Cell Biol.* **3**, 498508 (2002).
5. Boudreau, L. H. et al. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood* **124**, 2173-2183 (2014).
6. Chapman, E. R. How does synaptotagmin trigger neurotransmitter release? *Annu. Rev. Biochem.* **77**, 615641 (2008).
7. Sudhof, T. C. & Rothman, J. E. Membrane fusion: grappling with SNARE and SM proteins. *Science* **323**, 474-477 (2009).

6. Does daratumimab prevent or enhance release of “super-MEVs” or other extracellular mito from MSCs?

Response: We appreciate the reviewer for proposing this interesting hypothesis. According to your suggestion, we introduced daratumumab to block CD38 on MSCs and examined whether this would affect the release of “Super-EV-Mito” or other extracellular mitochondria from MSCs. We treated MSCs with daratumumab concurrently with CAP/CD38 complexes[1]. The results showed that treatment with daratumumab reduced the release of Super-EV-Mito and other extracellular mitochondria from MSCs (Figure Supplementary Data 1A, Fig. SD1A). We speculated that this effect might be attributed to daratumumab binding to CD38 receptors on the MSC membrane, thereby inhibiting the enzymatic activity of the intracellular domain of CD38, which catalyzed the production of a calcium messenger (cyclic ADP-ribose, cADPR)[2,3]. Therefore, daratumumab reduced intracellular and mitochondrial calcium levels (Fig. SD1B, C). Based on this, we proposed that daratumumab might suppress the release of Super-EV-Mito and other extracellular mitochondria from MSCs by inhibiting the CD38/Ca^{2}+ signaling pathway. This discussion has been included in the revised manuscript.

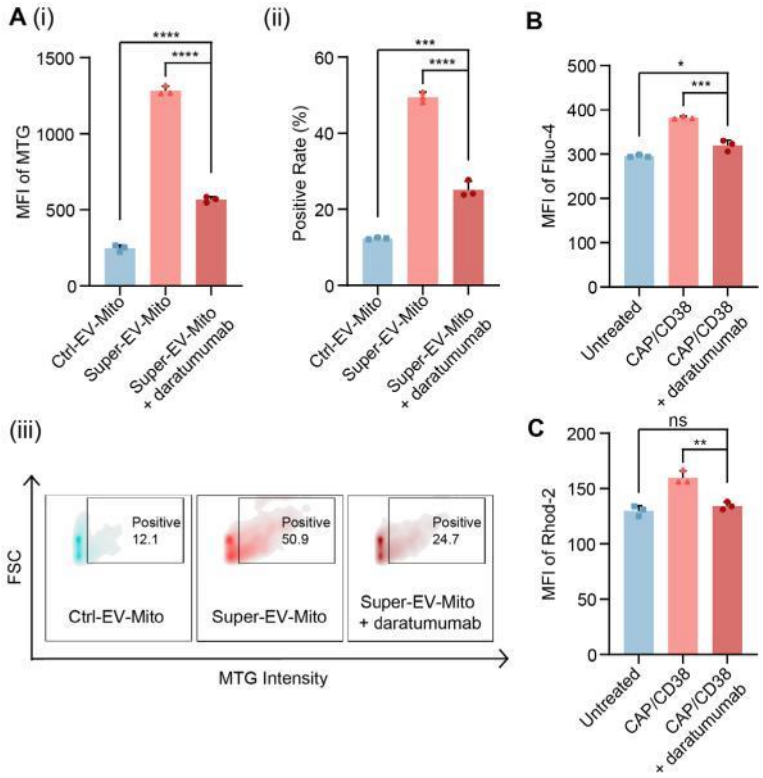

Fig. SD1. Daratumumab prevents the release of Super-EV-Mito and other extracellular mitochondria from MSCs. (A) Flow cytometry analysis of the MFI (i) and positive ratio (ii and iii) of MTG with or without daratumumab treatment. (B) The determination of intracellular Ca^{2+}. (C) The determination of mitochondrial Ca^{2+}.

1. Chakra, P. C. et al. Pre-clinical translational studies of daratumumab in patients with myeloma or AL amyloidosis undergoing autologous hematopoietic stem cell transplantation (SCT). *J. Clin. Oncol.* **33**, 8587-8587 (2015).
2. Kim, K. & Phelps, M. A. Clinical pharmacokinetics and pharmacodynamics of daratumumab. *Clin. Pharmacokinet.* **62**, 789-806 (2023).
3. Takasawa, S. CD38-cyclic ADP-ribose signal system in physiology, biochemistry, and pathophysiology. *Int. J. Mol. Sci.* **23**, 4306 (2022).

7. Figure 5 is just a cartoon with no data.

Response: We appreciate you pointing out this issue. To better present the content of the figure, we have renamed Figure 5 as Scheme 1 and relocated it to the end of all figures in the revised manuscript.

Thank you very much!

Reviewer #2 (Remarks to the Author):

This manuscript written by Wang et al. reported a potential method to promote the generation of mitochondria-enriched extracellular vesicles (MEVs) by MSCs through a genetic modification. They further show the therapeutic potential of their MEVs via the strategy of mitochondrial transfer. The work is of certain significance. However, the reviewer still has several concerns about the results and conclusions, and a further revision is necessary.

Response: Thank you for your kind words. We greatly appreciate your valuable comments. In response to your concerns, we have performed additional experiments and have added related explanations. Please find our responses to the comments point-by-point below.

1. The correlation between CD38 expression and MEV release has already been reported in previous studies (e.g. PMID: 36754021, 27466127). In this manuscript, authors applied this signal pathways to promote the production of MEVs via gene transfection using their previous reported gene vectors. Hence, the novelty of the present study in comparison with previous studies is suggested to further address.

Response: Thank you very much for pointing out this important issue. As the reviewer noted, the correlation between CD38 expression and extracellular vesicles containing mitochondria (EV-Mito) release has been documented in previous studies. Notably, PMID: 36754021 first reported that osteogenic induction stimulated mitochondrial fragmentation, donut formation, and the secretion of mitochondria via CD38/cADPR signaling[1]. PMID: 27466127 investigated mitochondrial transfer between astrocytes and neurons, mediated through CD38-calcium signaling[2]. However, whether CD38 signaling functions in a similar manner across different cell types remains unclear. Moreover, existing studies have primarily focused on spontaneous intercellular mitochondrial transfer mediated by EV-Mito in vivo. Inspired by these findings, we sought to address two major challenges in mitochondria transplantation therapy—namely, the lack of targeting specificity and the rapid inactivation of isolated mitochondria—using an EV-Mito-based strategy. In this manuscript, we activated the CD38/IP3R/Ca^{2}+ pathway through a non-viral gene engineering approach, which enabled the generation of “super donor MSCs” that produced Super-EV-Mito with a threefold increase in yield compared to Ctrl-EV-Mito

derived from unmodified MSCs. Super-EV-Mito not only protected mitochondrial activity through the membrane structure of EVs but also selectively recognized damaged recipient cells for functional mitochondrial delivery. Our study elucidates the underlying mechanism by which the CD38/IP3R/Ca^{2}+ signaling pathway enhances EV-Mito release, and leverages this mechanism to provide a greater quantity and improved quality of mitochondrial material for enhanced mitochondria transplantation.

In response to the reviewer's comment regarding the gene delivery vectors reported in our previous studies, we would like to clarify that our earlier work primarily focused on the construction of these vectors and demonstrated their capability to achieve efficient and safe gene transfection in high-GSH tumor cells via non-lysosomal pathways[3]. However, gene delivery into stem cells such as MSCs remains a major challenge due to their slow proliferation rate and limited phagocytic capacity[4,5]. In this manuscript, we first attempted to transfect MSCs using the commercially available Lipo3000 reagent. However, this method resulted in limited transfection efficiency, more critically, it raised concerns regarding cytotoxicity and biosafety. Therefore, developing a safe and efficient approach for the genetic engineering of MSCs is an urgent and unmet need. Given that MSCs also exhibit high intracellular GSH levels, we sought to extend the application of our previously developed gene delivery system, CAP, to MSC transfection. Our results showed that CAP-mediated gene delivery enabled high-efficiency and low-toxicity transfection in MSCs, indicating its potential as a promising tool for engineering "super donor" MSCs (Fig. S5 and Fig. S6).

Therefore, our key innovations are as follows: (i) We have generated "super donor MSCs" capable of secreting large amounts of extracellular vesicles containing mitochondria (EV-Mito). This strategy leverages **the membrane structure of EV-Mito not only to preserve mitochondrial activity but also to enable mitochondria to recognize and target damaged cells**; (ii) This is an innovative approach that links CD38 to Ca^{2+} signaling through IP3R and demonstrates that **upregulation of CD38 enhances the yield of EV-Mito**; (iii) We have designed and developed a **MSC microenvironment-triggered vector material**, enabling efficient genetic engineering of MSCs and producing "super donor cells" for EV-Mito. According to your suggestion, we have refined and highlighted these key points in the revised manuscript.

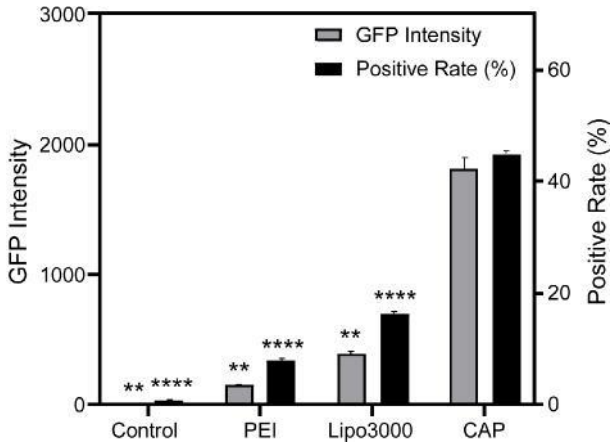

Fig. S5. Transfection efficiency of CAP/DNA complexes (w/w = 30/1) in MSCs. *n* = 3. Statistical analyses were performed using one-way ANOVA followed by Tukey's HSD post hoc test for comparisons of positive rates, or the Games-Howell test for GFP intensity. All significance comparisons were conducted relative to the CAP group.

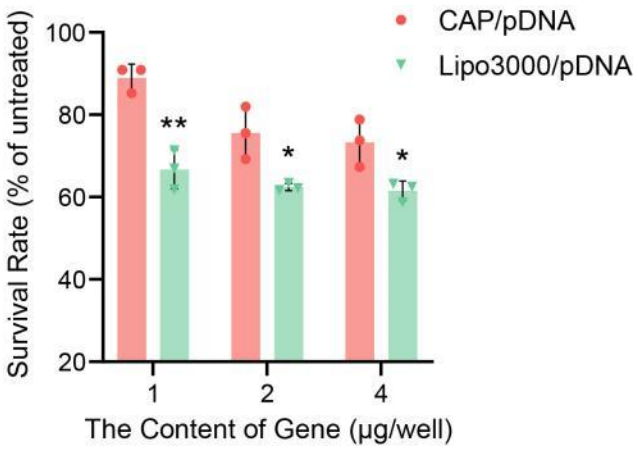

Fig. S6. The relative cell viability after treated with CAP/pDNA or Lipo3000/pDNA measured by MTT assay. *n* = 3. Statistical significances were performed via T-test. All significance comparisons were conducted relative to the CAP/pDNA group in different content of gene.

1. Suh, J. et al. Mitochondrial fragmentation and donut formation enhance mitochondrial secretion to promote osteogenesis. *Cell Metab.* **35**, 345-360 (2023).
2. Hayakawa, K. et al. Transfer of mitochondria from astrocytes to neurons after stroke. *Nature* **535**, 551-555 (2016).
3. Qi, L. Y., Wang, Y., Hu, L. F., Zhao, P. S. & Jiang, H. L. Enhanced nuclear gene delivery via integrating and streamlining intracellular pathway. *J. Control. Release* **341**, 511-523 (2022).
4. Jin, Y. et al. Serum-tolerant polymeric complex for stem-cell transfection and neural differentiation. *Nat. Commun.* **16**, 2022 (2025).
5. Stewart, M. P. et al. In vitro and ex vivo strategies for intracellular delivery. *Nature* **538**, 183-192 (2016).

2. The mechanisms by which intracellular Ca^{2+} regulates MEV production and how elevated Ca^{2+} levels promote MEV release are suggested to clarify.

Response: Thank you very much for pointing out this important issue. Previous studies have demonstrated that elevated intracellular $Ca^{2}+$ levels can trigger the fusion of vesicles with the plasma membrane through a Synaptotagmin/SNARE complex-mediated exocytosis mechanism, thereby promoting the release of extracellular vesicles (EVs) and their cargo[1,2]. Specifically, $Ca^{2}+$ elevation activates the calcium sensor Synaptotagmin on vesicles, inducing a conformational change that facilitates the assembly and activation of the SNARE complex, ultimately accelerating the membrane fusion and exocytosis of EV-Mito.

To elucidate the mechanism by which elevated intracellular calcium promotes EV release, we first confirmed that CD38-mediated $Ca^{2}+$ upregulation significantly increased EV-Mito secretion (Fig. 1E). Pharmacological inhibition studies further demonstrated that the release of EV-Mito was markedly suppressed by actin polymerization inhibitors, whereas microtubule inhibitors had no significant effect (Fig. S7C and D), suggesting that EV-Mito release was actin-dependent but independent of microtubule dynamics[3]. Previous studies have shown that intracellular $Ca^{2}+$ elevation activates the calcium sensor Synaptotagmin, a key regulator of $Ca^{2}+$-dependent exocytosis. Synaptotagmin contains two conserved C2 domains (C2A and C2B) that mediate $Ca^{2}+$-dependent interactions with phospholipids and SNARE proteins[4]. Upon $Ca^{2}+$ binding, these C2 domains undergo conformational changes that enable Synaptotagmin to interact with membrane lipids and facilitate the assembly of the SNARE complex, composed of proteins such as SNAP-25, syntaxin, and VAMP. The SNARE complex mediates the docking and fusion of vesicles with the plasma membrane, ultimately promoting EV release[5]. Taken together with our data, it could be speculated that CD38-induced $Ca^{2}+$ elevation enhanced EV-Mito production through a Synaptotagmin/SNARE complex-mediated exocytosis mechanism, which was tightly regulated by actin polymerization. This mechanistic insight highlighted a calcium-sensitive, actin-dependent vesicle trafficking pathway as a key contributor to EV-Mito secretion. In accordance with the reviewer's suggestion, we have strengthened the relevant experimental evidence and expanded the corresponding discussion in the revised manuscript.

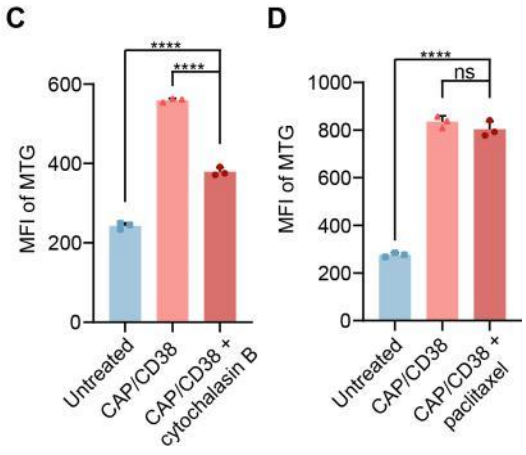

Fig. S7. IP3R protein expression changes in MSCs following CD38 transfection and the release of EV-Mito under different inhibitors. (C) The release of EV-Mito with or without actin inhibitors (cytochalasin B). (D) The release of EV-Mito with or without tubulin inhibitor (paclitaxel). *n* = 3. Statistical significances were performed via one-way ANOVA with Tukey's HSD post hoc test (C, D)

1. Kaeser, P. S. et al. RIM proteins tether Ca^{2+} channels to presynaptic active zones via a direct PDZ-domain interaction. *Cell* **144**, 282-295 (2011).
2. Chapman, E. R. Synaptotagmin: a Ca^{2+} sensor that triggers exocytosis? *Nat. Rev. Mol. Cell Biol.* **3**, 498508 (2002).
3. Boudreau, L. H. et al. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood* **124**, 2173-2183 (2014).
4. Chapman, E. R. How does synaptotagmin trigger neurotransmitter release? *Annu. Rev. Biochem.* **77**, 615641 (2008).
5. Sudhof, T. C. & Rothman, J. E. Membrane fusion: grappling with SNARE and SM proteins. *Science* **323**, 474-477 (2009).

3. As mentioned by the authors, the high quality of mitochondria is important. In this case, why the authors used MSCs as the donor cell of MEV, which usually the amount of mitochondria is limited? Will it be more effective by using the donor cell with more active mitochondria, such as myocardial cells?

Response: We thank the reviewer for raising this interesting question. We selected mesenchymal stem cells (MSCs) as donor cells for EV-Mito production because MSCs are metabolically inclined toward glycolysis and exhibit low intrinsic energy demands in their quiescent state, relying minimally on mitochondrial oxidative phosphorylation. As a result, they are more predisposed to donating mitochondria to the extracellular environment, facilitating

mitochondria transfer[1]. Meanwhile, accumulating evidences suggest that the paracrine effects of stem cells, including the extracellular release of mitochondrial particles, constitute a key mechanism underlying their ability to repair damaged cells[2]. Therefore, stem cells are among the most commonly used donor cells in mitochondria transfer studies[3,4].

According to the reviewer's suggestion, we examined EV-Mito release from different donor cell types. As shown in Figure supplementary data1 (Fig. SD1), when MSCs were used as donor cells, the mitochondrial positivity rate was 49.47 ± 1.40% after CAP/CD38 treatment, which was significantly higher than that observed when myocardial cells (AC16, human cardiomyocyte cell line) were used as donors after CAP/CD38 treatment (10.94 ± 0.48%). Even in the absence of CAP/CD38 treatment, the release of EV-Mito from MSCs remained significantly higher than that from AC16 cells. Although myocardial cells possess intrinsically higher mitochondrial activity, the result indicate that MSCs exhibit a greater capacity for EV-Mito release upon CD38 upregulation, making them a more favorable donor cell type under these conditions.

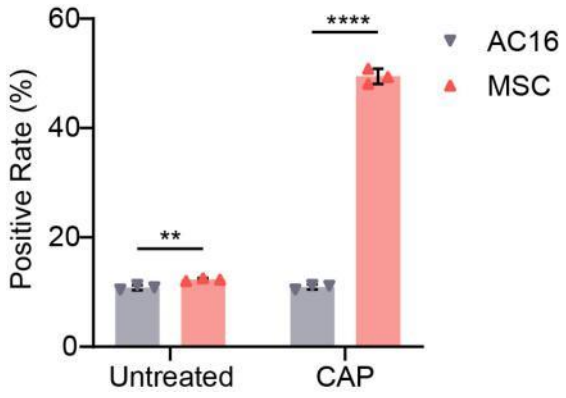

Fig. SD1. The percentage of Mitotracker Green-positive mitochondria in EV-Mito released from MSCs and AC16 cells was quantified by flow cytometry.

1. Huang, T. et al. Efficient intervention for pulmonary fibrosis via mitochondrial transfer promoted by mitochondrial biogenesis. *Nat. Commun.* **14**, 5781 (2023).
2. Malekpour, K., Hazrati, A., Soudi, S. & Hashemi, S. M. Mechanisms behind therapeutic potentials of mesenchymal stem cell mitochondria transfer/delivery. *J. Control. Release* **354**, 755-769 (2023).
3. Huang, T., Zhang, T. & Gao, J. Q. Iron oxide nanoparticles augment the intercellular mitochondrial transfer-mediated therapy. *Sci. Adv.* **7**, eabj0534 (2021).
4. Morrison, T. J. et al. Mesenchymal stromal cells modulate macrophages in clinically relevant lung injury models by extracellular vesicle mitochondrial transfer. *Am. J. Respir. Crit. Care Med.* **196**, 1275-1286 (2017).

4. It was previous reported that the package of impaired mitochondria in EVs is one of the approaches to eliminate the dysfunctional mitochondria (PMID: 34418352; 37108168). Therefore, whether the promotion of MEV will lead to more damaged mitochondria in EVs or how the authors to avoid the package of impaired mitochondria in their MEV?

Response: Thank reviewer for the valuable question. As the reviewer rightly pointed out, the packaging of impaired mitochondria into EVs is one of the cellular mechanisms for eliminating dysfunctional mitochondria. However, it is important to note that the extracellular release of damaged mitochondria typically occurs in response to mitochondrial injury. For example, PMID: 34418352 demonstrated that adipocytes respond to mitochondrial stress by rapidly releasing small extracellular vesicles containing damaged mitochondrial components[1]. Additionally, PMID: 37108168 reviewed mitochondria-containing extracellular vesicles release under oxidative stress, but also emphasized that in the absence of oxidative stress, mitochondria-containing extracellular vesicles can carry functional mitochondria, which may serve to rescue stressed recipient cells by restoring mitochondrial function[2].

In this manuscript, we collected Super-EV-Mito enriched with functional mitochondria under non-oxidative stress conditions. In response to the reviewer's concern, we examined the mitochondrial activity and functionality within EV-Mito. Specifically, we assessed oxygen consumption rate (OCR) using Seahorse analyzer. Extracted free mitochondria from normal MSCs were used as a control group. The result showed that the OCR per unit of mitochondrial protein was elevated in the Super-EV-Mito group compared to the extracted mitochondria (Fig. 2I). These findings further supported that CD38 activation not only promoted the release of EVs but also enhanced the function of mitochondria within Super-EV-Mito. We have incorporated the relevant results and corresponding explanations into the revised manuscript.

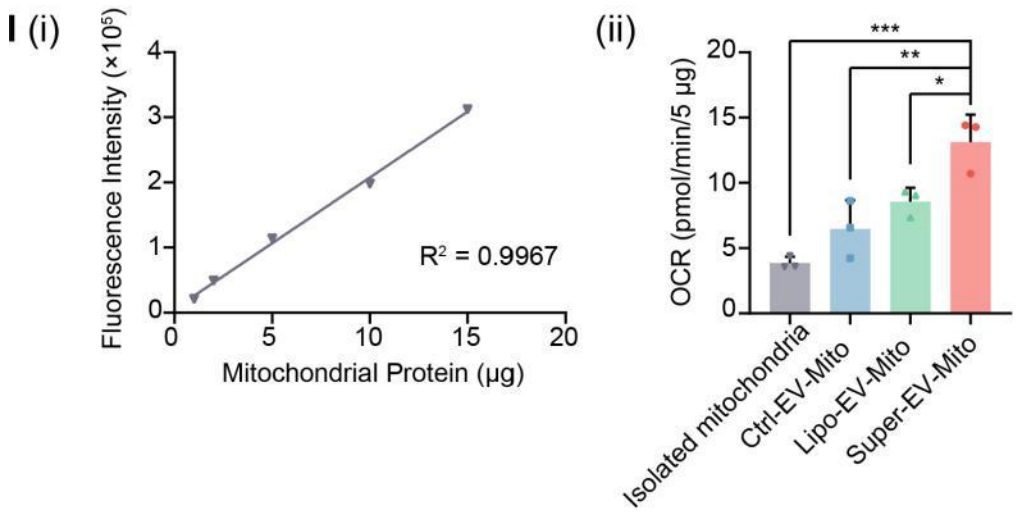

Fig. 2I. Preparation and characterizations of Super-EV-Mito. (I) OCR from Seahorse cell mito stress test of different EV-Mito. i: Standard curve between mitochondrial protein and fluorescence intensity of Mitotracker Green probe; ii: OCR normalized by mitochondrial protein in different EV-Mito groups.

1. Crewe, C. et al. Extracellular vesicle-based interorgan transport of mitochondria from energetically stressed adipocytes. *Cell Metab.* **33**, 1853-1868 (2021).
2. Sanz-Ros, J. et al. The potential use of mitochondrial extracellular vesicles as biomarkers or therapeutical tools. *Int. J. Mol. Sci.* **24**, 7005 (2023).

5. As introduced by the authors that the primary cause of LHON is the G11778A mitochondrial DNA mutation. Will the treatment using MEV repair this mitochondrial DNA mutation？If not, what is the therapeutic mechanism underlying the mitochondrial transfer? Also, if the administration of MEV is terminated, will the symptom of LHON recovered. More studies to figure out this issue is suggested, especially the long-term therapeutic potentials after the MEV administration.

Response: We sincerely thank the reviewer for this insightful comment. In mitochondrial diseases, mutated mtDNA often coexists with wild-type mtDNA in a condition known as heteroplasmy[1,2]. The severity of diseases caused by heteroplasmic mtDNA mutations is closely related to the mutant mtDNA load. A well-established threshold effect exists, wherein symptoms typically manifest only when the proportion of mutant mtDNA exceeds a critical threshold. Therefore, strategies aimed at reducing the mutant mtDNA load are considered a promising therapeutic approach[3,4].

Mitochondria transfer offers such a possibility by introducing exogenous, functional mitochondria into cells harboring pathogenic mtDNA mutations-such as LHON cells carrying the G11778A mutation. This approach can restore mitochondrial function by two synergistic mechanisms: (1) supplementing wild-type mtDNA to dilute the mutant load and shift the heteroplasmic ratio (Fig. S12A, C), and (2) promoting mitophagy to selectively eliminate damaged mitochondria containing high levels of mutant mtDNA (Fig. 3L). Hence, the therapeutic effect of mitochondria transfer arises not from editing or repairing mutant mtDNA directly, but from rebalancing mitochondrial homeostasis through both mitochondrial supplementation and mitophagy activation. Based on this mechanism, it is reasonable to expect that EV-Mito could offer long-term therapeutic benefits.

In accordance with the reviewer's suggestion, we further investigated the long-term therapeutic potential of Super-EV-Mito by examining both their persistence in GM10742 cells and their sustained ability to restore mitochondrial function. Our results showed that mtDNA originating from donor rat mitochondria could persist in GM10742 cells for up to 5 days post-administration (Fig. S12A). Furthermore, a single administration of Super-EV-Mito led to significantly improved mitochondrial function in GM10742 cells, which was maintained for up to 5 days (Fig. S12B). In addition, we examined the in vivo persistence of donor mtDNA in eye tissues for evaluate long-term therapeutic potential. After a single intraocular administration of Super-EV-Mito, donor rat mtDNA was detected for up to 14 days (Fig. S12C). These findings indicate that the transferred mitochondria retain functionality over an extended duration, underscoring the long-term therapeutic potential of Super-EV-Mito. Following the reviewer's recommendation, we have integrated the relevant experimental data and discussion into the revised manuscript to enhance clarity and completeness.

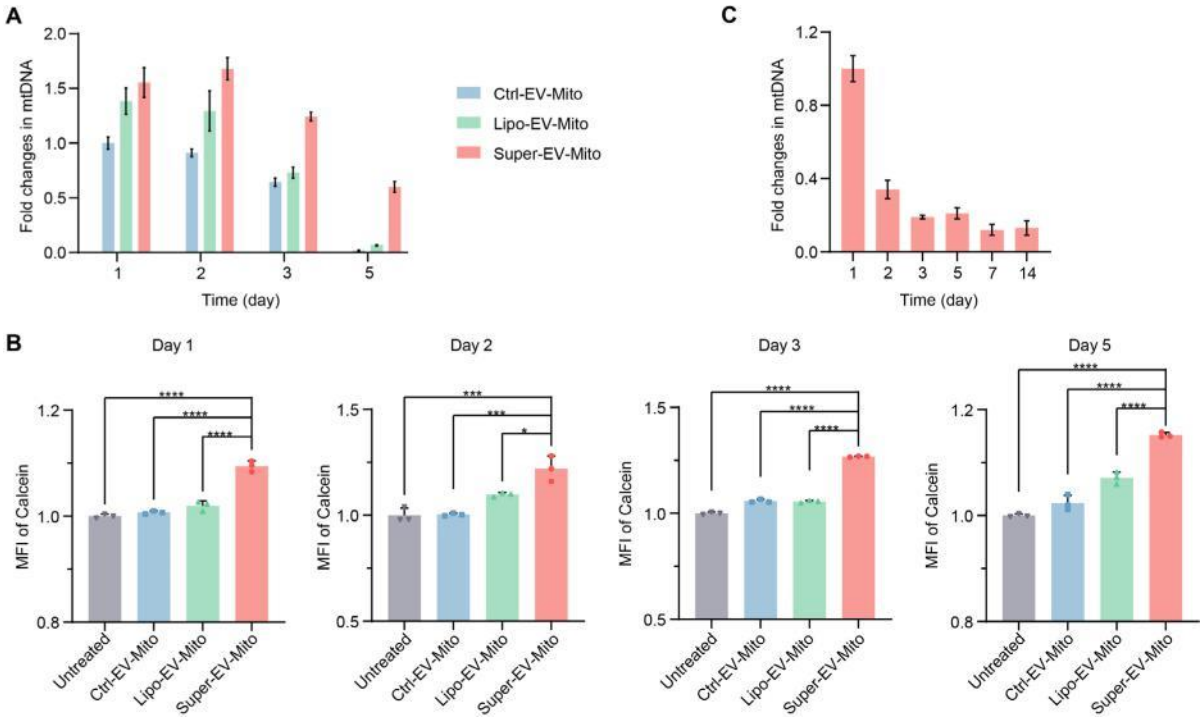

Fig. S12. Single-dose expression duration of Super-EV-Mito. (A) Time-course analysis of rat-derived mtDNA persistence in GM10742 cells following treatment with different types of EV-Mito. (B) Evaluation of the long-term mitochondrial permeability transition pore (mPTP) repair potential in GM10742 cells after a single administration of Super-EV-Mito. (C) Persistence of rat-derived mtDNA in mouse eyes following administration of Super-EV-Mito. n = 3. Statistical significances were performed via one-way ANOVA with Tukey's HSD post hoc test (B).

1. Stewart, J. B. & Chinnery, P. F. The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. *Nat. Rev. Genet.* **16**, 530-542 (2015).
2. Clyde, D. Mitochondrial DNA copy number and disease. *Nat. Rev. Genet.* **23**, 136 (2022).
3. Bacman, S. R. et al. MitoTALEN reduces mutant mtDNA load and restores tRNAAla levels in a mouse model of heteroplasmic mtDNA mutation. *Nat. Med.* **24**, 1696-1700 (2018).
4. Gammage, P. A. et al. Genome editing in mitochondria corrects a pathogenic mtDNA mutation in vivo. *Nat. Med.* **24**, 1691-1695 (2018).

6. How about the stability of mitochondria in MEV?

Response: In response, we evaluated the mitochondrial function in EV-Mito at different time points to reflect the stability of mitochondria within EV-Mito. As shown in Fig. 2J, the basal respiration measured by OCR did not show significant changes within two days. The result suggested that mitochondria encapsulated in EV-Mito maintained functional stability for at least two days.

We speculated that this stability was mainly attributed to the membrane structure of Super-EV-Mito, which protected mitochondrial activity. To investigate this hypothesis, we compared the mitochondrial quality in Super-EV-Mito and isolated mitochondria under conditions of high extracellular Ca^{2}+. The Mitotracker Red (MTR) probe was used for monitoring the changes of mitochondrial membrane potential (MMP). The result indicated that the MMP of isolated mitochondria was significantly compromised under this stress condition, whereas the MMP in Super-EV-Mito remained well preserved (Fig. S10). This suggested that the membrane structure of Super-EV-Mito effectively shielded mitochondria from damage caused by adverse extracellular environments. In response to the reviewer's suggestion, the revised manuscript has included the experimental result and accompanying discussion.

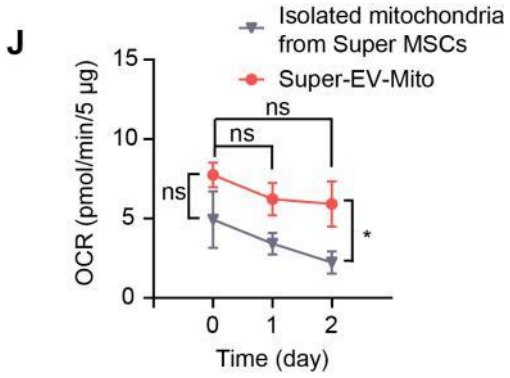

Fig. 2. Preparation and characterizations of Super-EV-Mito. (J) The stability of mitochondria in EV-Mito by monitoring changes in mitochondrial basal respiration.

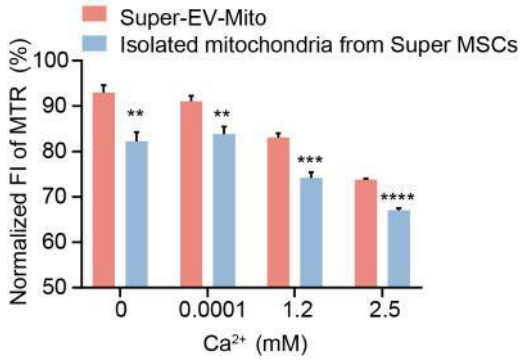

Fig. S10. The stability of mitochondria in Super EV-Mito under Ca^{2+} conditions. MTR intensity of Super-EV-Mito and isolated mitochondria incubated in different concentrations of Ca^{2+}. *n* = 3. Statistical significances were performed via T-test. All significance comparisons were conducted relative to the Super-EV-Mito group under different Ca^{2+} concentrations.

7. The quality of the mitochondria in super-MEV and super-donor MSCs is suggested to compare.

Response: We thank the reviewer for the constructive suggestion. In response, we evaluated the quality of mitochondria in both Super-EV-Mito and Super-donor MSCs using Seahorse assays[1,2]. As shown in Fig. SD2, there was no significant difference in basal respiration per unit mitochondrial protein between the Super-EV-Mito and Super-donor MSC groups. The result indicated that the mitochondrial quality was comparable between Super-EV-Mito and their donor cells, supporting the potential application of Super-EV-Mito in the treatment of mitochondrial diseases. To address the reviewer's concern, we have incorporated both the experimental findings and the corresponding discussion into the revised manuscript.

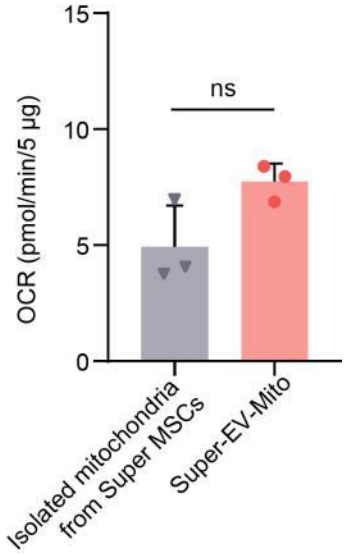

Fig. SD2. Assessment of mitochondrial respiratory function in Super-EV-Mito and Super-donor MSCs.

1. Mori, M. P. et al. Mitochondrial membrane hyperpolarization modulates nuclear DNA methylation and gene expression through phospholipid remodeling. *Nat. Commun.* **16**, 4029 (2025).
2. Dumas, L. et al. RNA G-quadruplexes control mitochondria-localized mRNA translation and energy metabolism. *Nat. Commun.* **16**, 3292 (2025).

8. The Ca^{2}+ overload is often associated with mitochondrial damage, which could pose potential risks for MEV. While the study examined changes in mitochondrial membrane structure in CAP/CD38-modified MSCs and reported that elevated Ca^{2}+ levels significantly suppressed mPTP opening compared to untreated MSCs, this observation appears inconsistent with

existing literature suggesting that Ca^{2}+ elevation promotes mPTP opening (PMID: 36410526, 31944918). Please discuss.

Response: Thank reviewer for the valuable question. In response to your suggestion, we have carefully reviewed and re-evaluated the design of the mPTP opening assay. Based on the fundamental principles of the mPTP assay, we found that this method may not be suitable for assessing whether CAP/CD38-mediated Ca^{2}+ elevation induces mitochondrial damage in MSCs, which could potentially pose risks for EV-Mito. This is because treatment with the CAP/CD38 complex significantly elevates intracellular Ca^{2}+ levels in MSCs. The increased Ca^{2}+ can competitively interfere with the cobalt quenching of calcein fluorescence, which is the core mechanism of the mPTP opening assay[1]. As a result, the observed elevation in calcein signal in CAP/CD38-treated MSCs compared to the untreated group may reflect a false-positive outcome, rather than an accurate inhibition of mPTP opening. Therefore, the mPTP opening assay might not reliably reflect mitochondrial function under conditions of altered intracellular calcium homeostasis.

As the reviewer correctly pointed out, CD38 upregulation can lead to elevated intracellular Ca^{2}+ levels. While Ca^{2}+ is essential for mitochondrial function, Ca^{2}+ overload is known to cause mitochondrial damage[2,3]. To more accurately assess the effects of CAP/CD38 treatment on mitochondrial integrity, we opted to use mitochondrial membrane potential (MMP) measurements rather than calcein-AM staining, which is more susceptible to interference from changes in mitochondrial content. As shown in Fig. 1L, the ratio of mitochondria with normal MMP to those with depolarized MMP in CAP/CD38-treated MSCs was significantly higher than that in untreated MSCs, indicating that CD38-mediated Ca^{2}+ elevation did not compromise mitochondrial membrane integrity.

To further verify whether CAP/CD38 treatment may pose potential risks to mitochondrial function in donor MSCs or EV-Mito, we examined the basal respiration levels of mitochondria in both CAP/CD38-treated MSCs and Super-EV-Mito using Seahorse analysis (Fig. 1J and Fig. 2I). The results demonstrated that the basal respiration levels of mitochondria were significantly increased following CAP/CD38 treatment, suggesting that moderate Ca^{2}+ elevation actually enhanced mitochondrial metabolic function.

Based on these findings, we propose that moderate $Ca^{2}+$ upregulation is beneficial and necessary for sustaining mitochondrial bioenergetics, and that only $Ca^{2}+$ overload would lead to dysfunction. Therefore, CAP/CD38 treatment, while elevating intracellular $Ca^{2}+$, does not impair but instead supports mitochondrial function, thereby minimizing the potential risks to the therapeutic efficacy of EV-Mito. Accordingly, we have incorporated MMP and Seahorse data into the revised manuscript to better illustrate that CD38-mediated $Ca^{2}+$ elevation does not compromise mitochondrial quality.

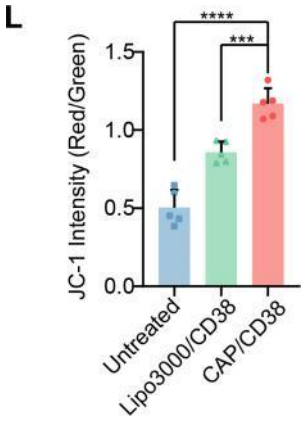

Fig. 1. CD38 signal increases mitochondrial calcium via IP3R and enhances extracellular mitochondria. (L) MMP detection.

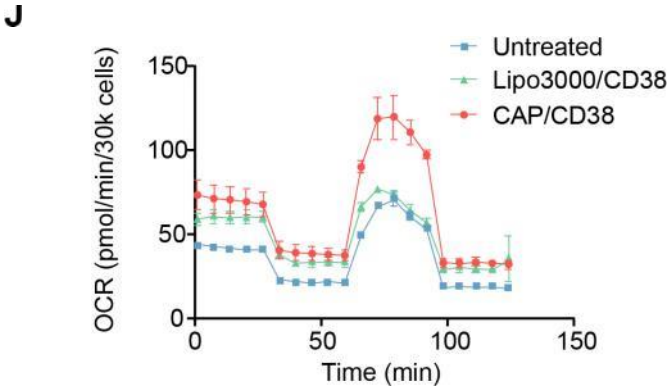

Fig. 1. CD38 signal increases mitochondrial calcium via IP3R and enhances extracellular mitochondria. (J) Oxygen consumption rate (OCR) from Seahorse cell mito stress test of MSCs after CD38 transfection.

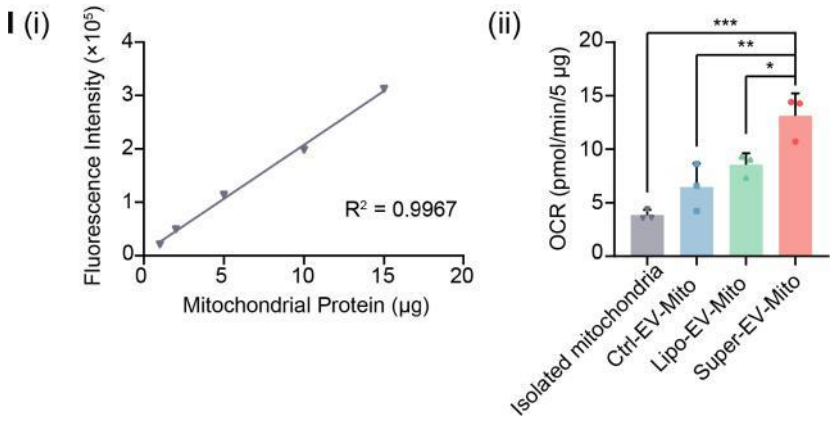

Fig. 2. Preparation and characterizations of Super-EV-Mito. (I) OCR from Seahorse cell mito stress test of different EV-Mito. i: Standard curve between mitochondrial protein and fluorescence intensity of Mitotracker Green probe; ii: OCR normalized by mitochondrial protein in different EV-Mito groups.

1. Sabbah, H. N. et al. Long-term therapy with Bendavia (MTP-131), a novel mitochondria-targeting peptide, reverses mitochondrial abnormalities in left ventricular myocardium of dogs with advanced heart failure. *Eur. Heart J.* **34**, P640 (2013).
2. Giorgi, C., Marchi, S. & Pinton, P. The machineries, regulation and cellular functions of mitochondrial calcium. *Nat. Rev. Mol. Cell Biol.* **19**, 713-730 (2018).
3. Gomez-Valades A. G. et al. Mitochondrial cristae-remodeling protein OPA1 in POMC neurons couples Ca^{2+} homeostasis with adipose tissue lipolysis. *Cell Metab.* **33**, 1820-1835 (2021).

9. In Fig1k, why there is no difference between Lipo3000 and CAP regarding to the basal respiration but has difference in terms of maximum respiration?

Response: We thank the reviewer for raising this important point. Basal respiration refers to the oxygen consumption rate (OCR) of cells under normal conditions, prior to the addition of compounds such as FCCP (a mitochondrial uncoupler)[1]. It reflects the baseline energy demand required to sustain essential cellular activities. In contrast, maximum OCR is measured after FCCP treatment, which disrupts the mitochondrial inner membrane proton gradient and forces the electron transport chain to operate at full capacity[2]. Thus, maximum OCR represents the maximal respiratory capacity or "stress response potential" of mitochondria.

In this study, the similar basal OCR levels observed between the Lipo3000 and CAP groups suggested that the mitochondrial quantity and basal function in MSCs were similar following either treatment. However, the reduced maximal OCR in the Lipo3000 group indicated an impaired mitochondrial stress response capacity, likely resulting from the known

cytotoxicity and safety concerns associated with Lipo3000 (Fig. S6). These findings further highlighted the superior safety profile of CAP as a gene delivery vector, particularly for mitochondrial applications, compared to conventional transfection reagents such as Lipo3000. In response to the reviewer's suggestion, this discussion has been added to the revised manuscript accordingly.

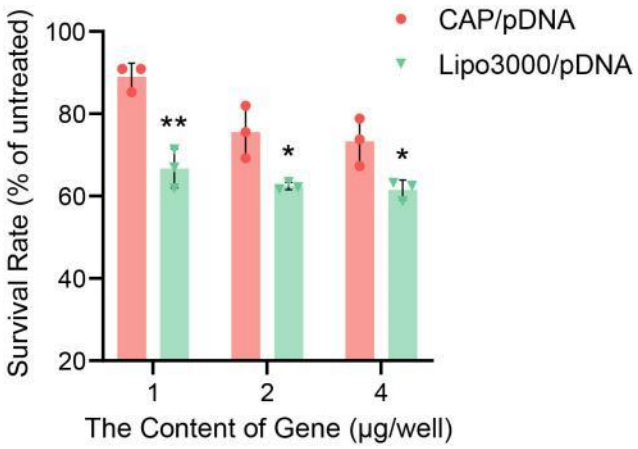

Fig. S6. The relative cell viability after treated with CAP/pDNA or Lipo3000/pDNA measured by MTT assay. *n* = 3. Statistical significances were performed via T-test. All significance comparisons were conducted relative to the CAP/pDNA group in different content of gene.

1. Takata, N. et al. Lactate-dependent transcriptional regulation controls mammalian eye morphogenesis. *Nat. Commun.* **14**, 4129 (2023)..
2. Mezhevova, I. V. et al. Some berberine molecule modifications that abrogate its inhibitory effect on cell oxidative phosphorylation. *J. Clin. Oncol.* **40**, e15078-e15078 (2022).

10. More proofs are required to support the conclusion that the mitochondria in super-MEV are functional, by saying "higher content of functional mitochondria in Super-MEVs (Fig. 3A, B)." The current data can support more content of mitochondria, but hardly to say more functional mitochondria.

Response: We thank the reviewer for the valuable suggestion. According to your comment, we investigated whether Super-EV-Mito contains more functional mitochondria by assessing the basal respiration. As shown in the Fig. 2I, the basal respiration per unit mitochondrial protein in Super-EV-Mito was significantly higher than that in Ctrl-EV-Mito and Lipo3000-EV-Mito. These findings indicated that, even at equal mitochondrial proteins, the mitochondria encapsulated within Super-EV-Mito exhibited superior basal respiration function. The

experimental results and relevant discussion have been added to the revised version in response to the reviewer's feedback.

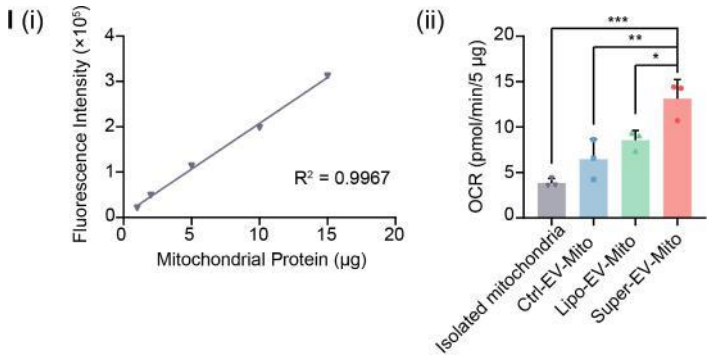

Fig. 2. Preparation and characterizations of Super-EV-Mito. (I) OCR from Seahorse cell mito stress test of different EV-Mito. i: Standard curve between mitochondrial protein and fluorescence intensity of Mitotracker Green probe; ii: OCR normalized by mitochondrial protein in different EV-Mito groups.

11. Similarly, in the evaluation of the protective effects of MEVs on mitochondria, the authors used MTG fluorescence intensity as an indicator of mitochondrial changes. However, this approach lacks characterization of mitochondrial functional alterations under different stress conditions. Additionally, in the stimulation experiments, the rationale for selecting the Ca^{2}+ concentration gradient (0, 0.0001, 1.25, 2.5 mM) remains unclear and requires further clarification.

Response: We sincerely thank the reviewer for this insightful suggestion. In response to the reviewer's comment, we assessed changes in mitochondrial membrane potential (MMP) using MMP-dependent Mitotracker Red probe (MTR) to characterize mitochondrial functional alterations under high Ca^{2+} level. Because MMP is a critical indicator of mitochondrial function, as it drives ATP synthesis, metabolite transport, calcium uptake, and other essential mitochondrial processes[1,2]. As shown in Fig. S10, MMP in isolated mitochondria was significantly reduced under Ca^{2}+ treatment, indicating mitochondrial dysfunction. In contrast, the mitochondria encapsulated within Super-EV-Mito maintained relatively stable MMP, suggesting that the protective membrane structure of Super-EV-Mito effectively shields mitochondria from external stress-induced damage.

Regarding the Ca^{2}+ concentration gradient (0, 0.0001, 1.25, and 2.5 mM), the rationale for selecting these calcium concentrations was to simulate the mitochondrial functionality of Super-EV-Mito under cytosolic, extracellular, and blood calcium conditions, respectively. Specifically, 0.0001 mM corresponds to cytosolic calcium levels, 1.25 mM reflects typical extracellular calcium concentrations, and 2.5 mM represents blood calcium levels[3]. As the rapid inactivation of isolated mitochondria in extracellular environments has been widely attributed to interference from elevated extracellular Ca^{2}+ concentrations, we employed this gradient based on previously reported physiological ranges to investigate mitochondrial function under these distinct conditions. To address the reviewer's concern, we have incorporated both the experimental findings and the corresponding discussion into the revised manuscript.

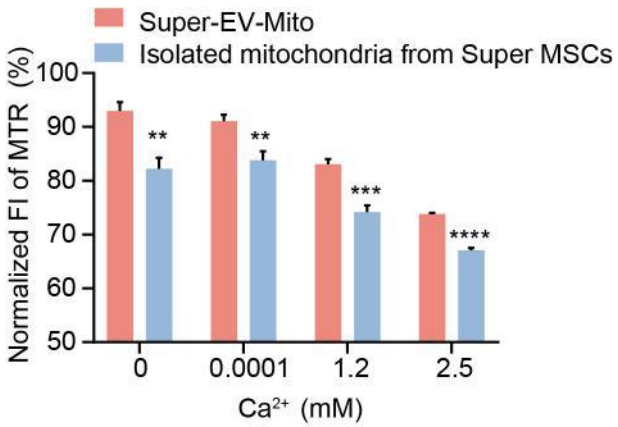

Fig. S10. The stability of mitochondria in Super EV-Mito under Ca^{2+} conditions. MTR intensity of Super-EV-Mito and isolated mitochondria incubated in different concentrations of Ca^{2+}. *n* = 3. Statistical significances were performed via T-test. All significance comparisons were conducted relative to the Super-EV-Mito group under different Ca^{2+} concentrations.

1. Sun, J. et al. Metabolic regulator LKB1 controls adipose tissue ILC2 PD-1 expression and mitochondrial homeostasis to prevent insulin resistance. *Immunity* **57**, 1289-1305 (2024).
2. Paracatu, L. C. et al. Stathmin-1 regulates hematopoietic stem cell mitochondrial and cellular function. *Blood* **142**, 4045 (2023).
3. Ikeda, G. et al. Mitochondria-rich extracellular vesicles from autologous stem cell-derived ccardiomyocytes restore energetics of ischemic myocardium. *J. Am. Coll. Cardiol.* **77**, 1073-1088 (2021).

12. "SuperMEV treatment increased COX IV expression compared with Ctrl-MEV treatment (Fig. 3C and Fig. S12B)". However, the data showed there is no statistic difference among

Super-MEVs, Ctrl-MEV and Lipo-MEV. Partially, there are several data including the therapeutic outcomes showed no statistic difference between Super-MEVs and Lip-MEV. What are the major advantages of CAP, except the biocompatibility?

Response: We appreciate the reviewer's insightful comments. As suggested, we re-evaluated the expression levels of mitochondrial genes mt-ND4 and COX IV in GM10742 cells after different treatments. The results showed that treatment with Super-EV-Mito significantly increased the expression of both mt-ND4 and COX IV compared to other groups. These results have been incorporated into the revised manuscript (Fig. 3B and Fig. S11). As the reviewer correctly pointed out, some indicators in Fig. 3 did not show a statistically significant difference between Super-EV-Mito and Lipo-EV-Mito. However, several key functional parameters, including ATP production and the recovery of GM10742 cell proliferation, were significantly improved by Super-EV-Mito treatment compared to Lipo-EV-Mito as shown in Fig. 3H, J and K.

The major advantage of CAP over Lipo3000 lies in its higher transfection efficiency in hard-to-transfect cells such as MSCs (as shown in Fig. S5). This enhanced efficiency facilitated the generation of Super-EV-Mito with superior mitochondrial functionality compared to Lipo-EV-Mito (Fig. 2I). Furthermore, due to the superior biosafety profile of CAP (Fig. S6), the mitochondria in CAP/CD38-treated MSCs exhibited a stronger maximal respiratory capacity compared to Lipo3000/CD38-treated MSCs (Fig. 1J).

In summary, safety and efficacy are the ultimate goals of gene delivery systems. CAP offers both higher transfection efficiency and better safety compared to Lipo3000, making it more suitable for the preparation of super donor cells and functional Super-EV-Mito.

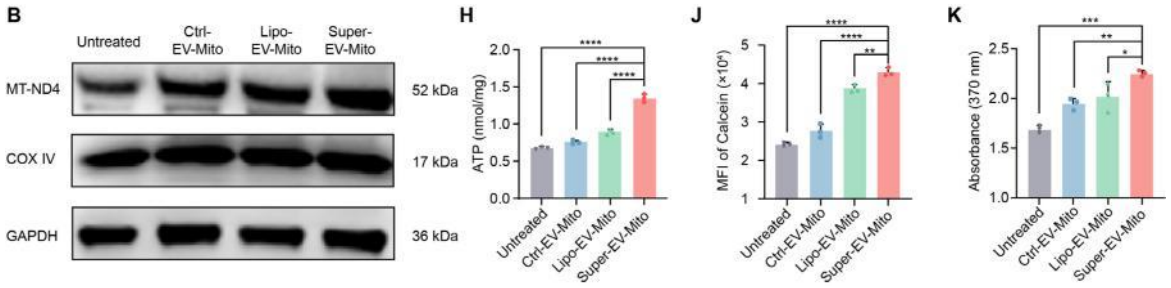

Fig. 3. Super-EV-Mito restore the mitochondrial functions of GM10742 cells. (B) The expression of MT-ND4 and COX IV protein detected by Western blot assay. (H) The ATP contents. (J) The mPTP opening detection. (K) Cell proliferation evaluated using EdU assay.

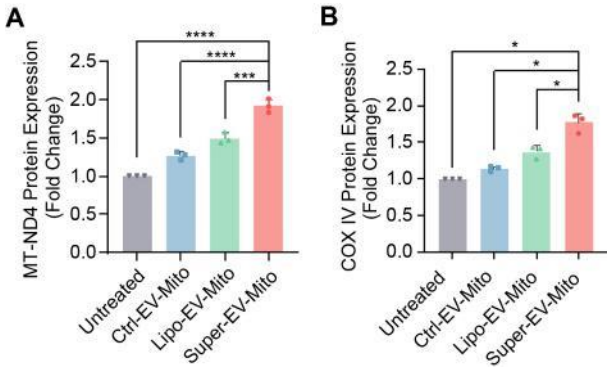

Fig. S11. Mitochondrial protein expression after different treatments in GM10742 cells. (A) MT-ND4 protein. (B) COX IV protein. *n* = 3. Statistical significances were performed via one-way ANOVA with Tukey's HSD post hoc test (A) or Games-Howell test (B).

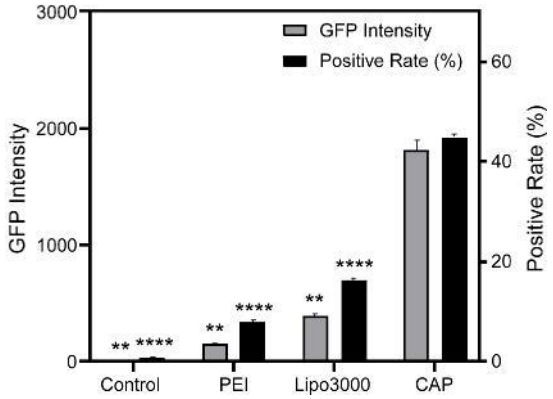

Fig. S5. Transfection efficiency of CAP/DNA complexes (w/w = 30/1) in MSCs. *n* = 3. Statistical significances were performed via one-way ANOVA with Tukey's HSD post hoc test (Positive rate) or Games-Howell test (GFP intensity).

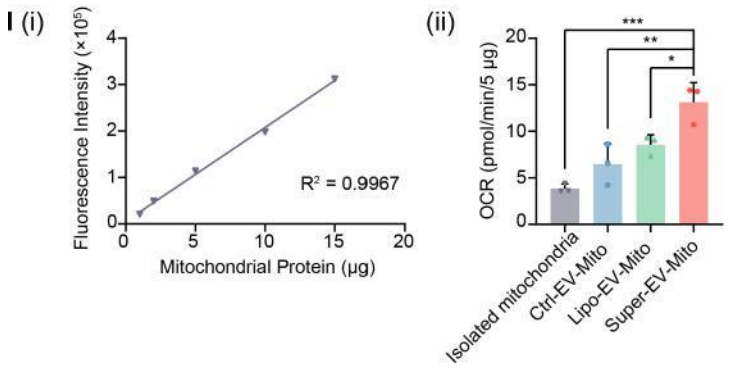

Fig. 2. Preparation and characterizations of Super-EV-Mito. (I) OCR from Seahorse cell mito stress test of different EV-Mito. i: Standard curve between mitochondrial protein and fluorescence intensity of Mitotracker Green probe; ii: OCR normalized by mitochondrial protein in different EV-Mito groups.

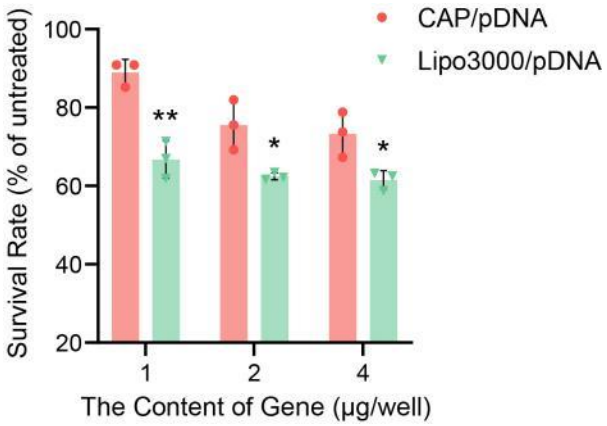

Fig. S6. The relative cell viability after treated with CAP/pDNA or Lipo3000/pDNA measured by MTT assay. *n* = 3. Statistical significances were performed via T-test. All significance comparisons were conducted relative to the CAP/pDNA group in different content of gene.

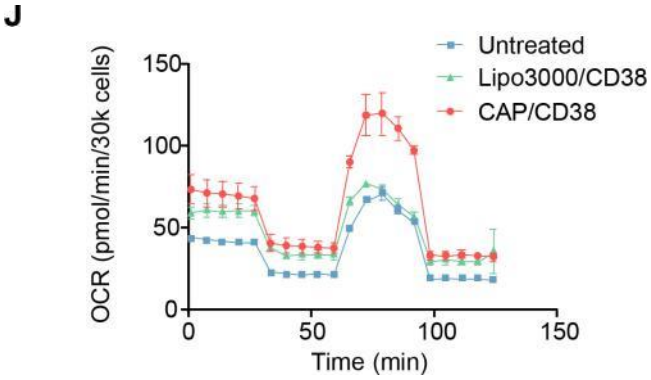

Fig. 1. CD38 signal increases mitochondrial calcium via IP3R and enhances extracellular mitochondria. (J) Oxygen consumption rate (OCR) from Seahorse cell mito stress test of MSCs after CD38 transfection.

13. Will the regulation of CD38/IP3R/Ca^{2}+ affect other functions of MSCs, such as the migration and differentiation?

Response: We thank the reviewer for raising this insightful question. In response to the reviewer's feedback, we further investigated whether CD38/IP3R/Ca^{2}+ signaling might indirectly affect MSC migration or differentiation by performing additional cell scratch assay and osteogenic differentiation assay (Fig. SD3). Our results showed that modulation of the CD38/IP3R/Ca^{2}+ axis partially inhibited MSC migration while promoting osteogenic differentiation, which is likely attributable to the enhanced mitochondrial activity and function induced by CD38 overexpression[1,2]. To minimize the potential risks associated with CAP/CD38 complex treatment, we ensured that each batch of MSCs was subjected to the treatment only once for EV-Mito collection. No repeated harvesting of EV-Mito from the same treated MSCs was performed. These experimental results and the corresponding discussion have been added to the revised manuscript accordingly.

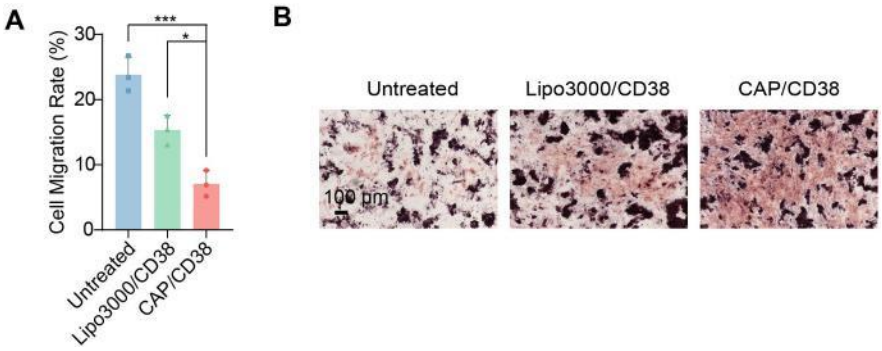

Fig. SD3. Mitochondrial functions in MSCs after different treatments. (A) The migration of MSCs. (B) The osteogenic differentiation of MSCs.

1. Döhla, J. et al. Metabolic determination of cell fate through selective inheritance of mitochondria. *Nat. Cell Biol.* **24**, 148-154 (2022).
2. Sun, B. et al. Actin polymerization state regulates osteogenic differentiation in human adipose-derived stem cells. *Cell. Mol. Biol. Lett.* **26**, 15 (2021).

14. Although several data demonstrated the statistic difference of Super MEVs in comparison with Ctril-MEVs or Lipo-MEVs, the increasing times of the values looks not so obviously. Please discuss it.

Response: Thank the reviewer for the comment. As noted by the reviewer, although several indicators—such as intracellular ATP levels and cell proliferation—showed statistically significant differences between Super-EV-Mito and Ctrl-EV-Mito or Lipo-EV-Mito, the fold-change increases appeared relatively modest. This may be attributed to the fact that mitochondria are the primary therapeutic agents in mitochondria transplantation, and thus their loading content directly affects therapeutic efficacy. Therefore, the extent of functional enhancement is expected to correspond with the mitochondrial content among Super-EV-Mito, Ctrl-EV-Mito, and Lipo-EV-Mito.

As shown in Fig. 2G(ii), the mitochondrial loading in Super-EV-Mito was approximately 3.87-fold and 1.22-fold higher than in Ctrl-EV-Mito and Lipo-EV-Mito, respectively. Correspondingly, intracellular ATP levels in the Super-EV-Mito group were 1.78-fold and 1.51-fold higher than in Ctrl-EV-Mito and Lipo-EV-Mito groups, respectively (Fig. 3H). In addition, cell proliferation in the Super-EV-Mito group was 1.16-fold and 1.12-fold higher than in the respective controls (Fig. 3K).

These findings suggested that the therapeutic efficacy of EV-Mito formulations is closely associated with their mitochondrial content. The significantly enhanced mitochondrial loading of Super-EV-Mito likely accounts for its superior performance in functional metrics, which exhibited a 1.5~4-fold or 1~1.5-fold increase compared to Ctrl-EV-Mito and Lipo-EV-Mito, respectively. In accordance with the reviewer's suggestion, we have included additional discussion on this point in the revised manuscript.

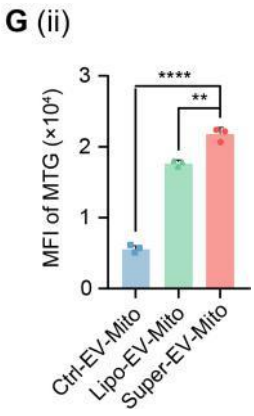

Fig. 2. Preparation and characterizations of Super-EV-Mito. (G) The MFI of MTG of EV-Mito in different treatment groups using flow cytometry analysis.

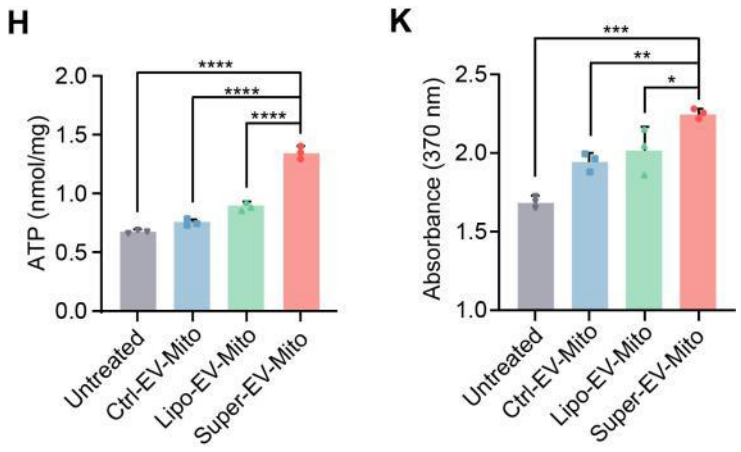

Fig. 3. Super-EV-Mito restore the mitochondrial functions of GM10742 cells. (H) The ATP contents. (K) Cell proliferation evaluated using EdU assay.

15. Fig.1c, the background of IP3R and GAPDH look so different. What is the reason?

Response: Thank you for pointing out this issue. The difference in background between IP3R and GAPDH in Fig. 1C was due to the distinct endogenous expression levels of these two proteins, which required different exposure times during imaging. Following your suggestion, we conducted repeated Western blot analyses with matched exposure times for both proteins. The consistency of the result confirmed that the initial finding was not influenced by exposure time differences. We have replaced the WB bands in Fig. 1C with the updated results in the revised manuscript.

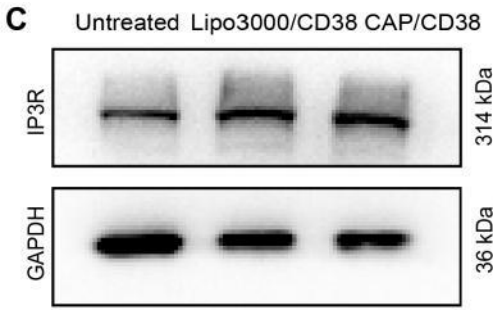

Fig. 1. CD38 signal increases mitochondrial calcium via IP3R and enhances extracellular mitochondria. (C) Gene expression of IP3R in MSCs after different treatments.

16. Fig.5 may be more suitable listed in the end of Fig.6.

Response: We appreciate you pointing out this issue. In response to the reviewer's suggestion, we have renamed Figure 5 as Scheme 1 and relocated it to the end of all figures in the revised manuscript.

Thank you very much!