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Short Communication

Cyclophilin D modulates mitochondrial bioenergetics, but its deficiency does not enhance cell viability following mitochondrial transplantation



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Introduction

The peptidylprolyl cis-trans isomerase Cyclophilin D (CyPD), encoded by the *PPIF* gene, is the only member of the Cyclophilin protein family present in the mitochondrial matrix [1, 2]. CyPD is indeed a key protein in the mitochondrial proteome and interacts with numerous protein partners, thus exerting diverse biological functions in the mitochondrial homeostasis. Nevertheless, the best characterized function of CyPD is the promotion of the permeability transition pore (mtPTP) opening by association with the actual pore component(s). The mtPTP is a multi-conductance Ca^{2+}-dependent channel of the mitochondrial inner membrane, whose long-lasting opening disrupts bioenergetics parameters leading to mitochondrial swelling and ultimately cell death. The current consensus model is that the mtPTP is mediated both by ATP synthase and the adenine nucleotide transporter [1].

The mtPTP involvement in pathology has been extensively documented, supporting the notion that CyPD could serve as a promising therapeutic target [1]. Dysregulated mtPTP opening is increasingly recognized as a pivotal contributor to cardiac injury, with mitochondrial transplantation emerging as a novel strategy for myocardial protection. This therapeutic approach involves isolating healthy, respiration-competent mitochondria from a donor and delivering them into the damaged cardiac tissue. The primary objective of our study was to test the effects on cell viability of transplanting mitochondria derived from either wild type or *PPIF-/-* HEK293 cells into pericytes isolated from donor cardiac atria. We hypothesized that *PPIF-/-* mitochondria, being less prone to mtPTP opening under calcium overload conditions potentially encountered during transplantation, would also exhibit improved bioenergetic performance through reduced CyPD-mediated inhibition of ATP synthase

[3]. Given the limited understanding of CyPD's role in mitochondrial bioenergetics, this study also addresses this critical knowledge gap by characterizing respiratory function in isolated mitochondria from HEK293 WT and *PPIF-/-* cells.

Methods

Mitochondria were isolated from WT and *PPIF-/-* HEK293 cells using the QIAGEN QMitochondria Isolation Kit. Oxygen consumption of freshly isolated mitochondria was evaluated through high-resolution respirometry (Oroboros Oxygraph-2K) adopting a substrate-uncoupler-inhibitor-titration protocol [4]. The maximal oligomycin-sensitive ATP hydrolytic capacity of ATP synthase was measured using the acidification channel (ECAR) of the Seahorse XFe24 Analyzer by sequentially adding 20 mM ATP and 5 µM FCCP to respiring mitochondria [5]. Pericytes isolated from cardiac atrial tissue of healthy donors (n = 6; 3 males and 3 females; age 38–63 years) were cultured in DMEM and treated for 24 h with WT or $PPIF^{-/-}$ mitochondria (25 µg/cm²). Cell viability was assessed using the MTT assay, while proliferation was evaluated by counting Hoechst-stained nuclei. The isolation and use of human pericytes were approved by the SC Health Technology Assessment and the Segreteria Comitato Etico Unico Regionale (Protocol No. 0000725, 08/01/2025).

Results and discussion

Although it is well established that *PPIF-/-* mitochondria display a much lower propensity for mtPTP opening [6], their respiration pattern remains poorly characterized. Oxygen consumption profiles obtained by high-resolution respirometry show that, with both CI and CII-linked substrates fueling respiration, mitochondria from *PPIF-/-* cells exhibit much higher ADP-stimulated (OXPHOS) and maximal noncoupled (ETS) respiration. Moreover, KO samples tend to exhibit a reduced LEAK respiration compared with WT, which is consistent with the mtPTP being significantly more sensitive to low Ca^{2+} in WT mitochondria (Fig. 1B). Such differential behavior could be explained by the following findings: i) In WT mitochondria, the NADH-linked pathway (through CI) is impaired (Fig. 1C), despite the similar CI protein levels in the two genotypes (data not shown); we hypothesize that the reduced CI-linked respiration is a consequence of NAD^+ depletion associated with mtPTP opening during mitochondria isolation [7], which ultimately affects all respiration states; ii) the relief of CyPD inhibitory effect on ATP synthase in *PPIF-/-* mitochondria leads to a higher ADP-stimulated respiration. Consistently, the maximal ATP hydrolytic capacity is about 30% higher in *PPIF*-/- samples than in WT mitochondria (Fig. 1D), as already observed [3]. Co-incubation of cardiac pericytes with mitochondria from both genotypes (Fig. 1E) leads to a significant increase in cell viability and proliferation, with no statistically significant difference between the two genotypes. These findings indicate that, contrary to our hypothesis, the improved bioenergetic profile of *PPIF-/-* mitochondria and their reduced sensitivity to Ca^{2+}-induced mtPTP opening did not translate into a greater functional benefit for pericytes after mitochondrial transplantation. Our working

hypothesis is that the bioenergetic fitness and/or mtPTP opening may not influence mitochondrial internalisation or intracellular retention following mitochondrial transplantation, although we cannot exclude that these two processes may be differently affected by CyPD. Further studies on models of cellular dysfunction are needed to assess whether the transplantation of *PPIF-/-* mitochondria could be beneficial under mtPTP-associated stresses.

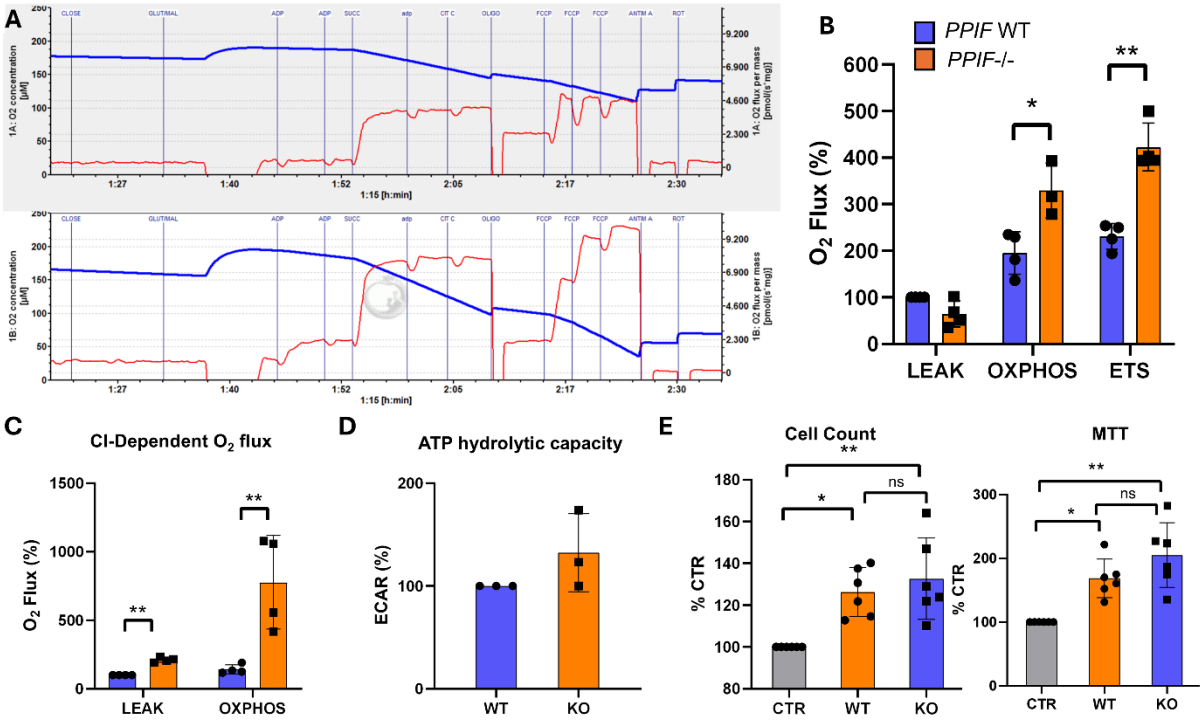


Figure 1. A) Representative high-resolution respirometry traces of mitochondria from WT (top trace) and *PPIF-/-* HEK293 cells (bottom trace). Experiments were performed in air-saturated respiration medium (MIR05) at 37°C [4]. **B)** Respiration rates in A, fueled with malate (4 mM), glutamate (10 mM) and succinate (10 mM), were corrected for residual O_2 consumption, normalized to protein content, and expressed relative to WT LEAK-state respiration, obtained after 4 µM oligomycin addition (100% for each biological replicate). **C)** Respiration values from A obtained with malate and glutamate only. Data are expressed as in B. **D)** The maximal oligomycin-sensitive ATP hydrolytic capacity of WT and *PPIF-/-* mitochondria was measured as acidification rates using the Seahorse XFe24 Analyzer. **E)** WT and *PPIF-/-* mitochondria were co-incubated with pericytes for 24 h, then MTT assay or cell counting through Hoechst staining were performed. Statistical analysis was performed using paired two-tailed t-tests in B-D (WT vs KO), and the Kruskal-Wallis test followed by Dunn's test in E (* $p < 0.05$; ** $p < 0.01$).

Conflicts of interest: The authors declare no conflict of interest.

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Author contributions: EZ, GCo, GCa performed the experiments and analyzed the data. MC contributed to data analysis and interpretation. GL, APB, and SS acquired funding. GL, GCo, and MC contributed to drafting the manuscript. GL conceptualized and supervised the work.

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