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LETTER TO THE EDITOR

High mitochondrial Ca^{2+} content increases cancer cell proliferation upon inhibition of mitochondrial permeability transition pore (mPTP)

Saverio Marchi[a], Veronica Angela Maria Vitto[a], Simone Patergnani[a,b], and Paolo Pinton[a,b]

[a]Department of Morphology, Surgery and Experimental Medicine, Section of Pathology, Oncology and Experimental Biology, Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, Ferrara, Italy; [b]Cecilia Hospital, GVM Care & Research, E.S: Health Science Foundation, Cotignola, Italy

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The Mitochondrial Calcium Uniporter (MCU) complex, the channel responsible for Ca^{2+} entry inside mitochondria, is involved in a wide range of diseases, including cancer [1]. Although elevated mitochondrial Ca^{2+} levels have been associated to apoptosis induction, probably by promoting the activation of mitochondrial Permeability Transition Pore (mPTP), a key effector of cell death [2], up-regulation of MCU complex functions have been observed in different cancer contexts [3]. Thus, it is unclear if increased mitochondrial Ca^{2+} content could sustain cancer progression or augment the susceptibility to apoptosis. Here, we show that high mitochondrial [Ca^{2+}] with concomitant mPTP inhibition could potentiate migratory capacity and invasiveness of cancer cells.

To test the impact of high basal mitochondrial Ca^{2+} levels on cancer-related features, we used HeLa cells stably silenced for *MICU1* gene [4] or PC3 prostate cancer cells stably overexpressing the pore-forming channel subunit MCU [5] (Figure 1(a)). Using a mitochondrial-targeted GCaMP6m probe, which displayed high Ca^{2+} sensitivity [4], we observed that both MICU1 depletion and MCU up-regulation induced an increase in mitochondrial [Ca^{2+}] at resting conditions (Figure 1(b–c)), due to the accumulation of MCU channel complexes that are not regulated by MICU activity [6,7]. Loss of the gatekeeping functions predisposed to cell death induced by the Ca^{2+}-dependent apoptotic stimulus C2-ceramide (Figure 1(d–e)), which is minimized by pre-treatment with the known mPTP inhibitor Cyclosporine A (CsA), thus suggesting that high MCU Ca^{2+} affinity sensitizes cells to apoptosis by affecting mPTP opening. Importantly, in our cellular settings, high mitochondrial Ca^{2+} levels do not promote cell death at basal conditions, indicating that the elevated capacity of mitochondria to accumulate Ca^{2+} is not toxic *per se*. Moreover, CsA treatment does not affect mitochondrial Ca^{2+} levels (Figure 1(b–c)). With our surprise, analysis of cancer cell proliferation, using a crystal violet-based assay, revealed that CsA treatment strongly augmented the proliferative rate of cells with high mitochondrial Ca^{2+} content, either due to MICU1 depletion (Figure 1(f)) or MCU over-expression (Figure 1(g)), without affecting the growth of the control counterparts.

To further dissect this event, we examined other cellular processes that are often associated with tumor progression and aggressiveness. Firstly, we measured the ability of both HeLa and PC3 to migrate through a basement membrane following a serum gradient using a Boyden chamber assay. Treatment with CsA significantly increased the capacity to cross the membrane exclusively in MICU1 Knock-Down (KD) HeLa or MCU-overexpressing PC3 cells (Figure 1(h–i)). Next, we assessed the combinatory effect of higher mitochondrial Ca^{2+} levels and mPTP inhibition on the capability to close the gap in a classical wound-healing assay. As shown in Figure 1(j), CsA treatment positively regulate MICU1 KD cells in the closure of the wound (% of open area after 24 h from the wound: 9.23 ± 3.83), whereas it does not affect the migration of control cells (plko +CsA: 54.23 ± 7.18). Similar results have been obtained in PC3 cells (Figure 1(k)), where CsA promotes the closure of scratched area only in

CONTACT Paolo Pinton paolo.pinton@unife.it; Saverio Marchi saverio.marchi@unife.it

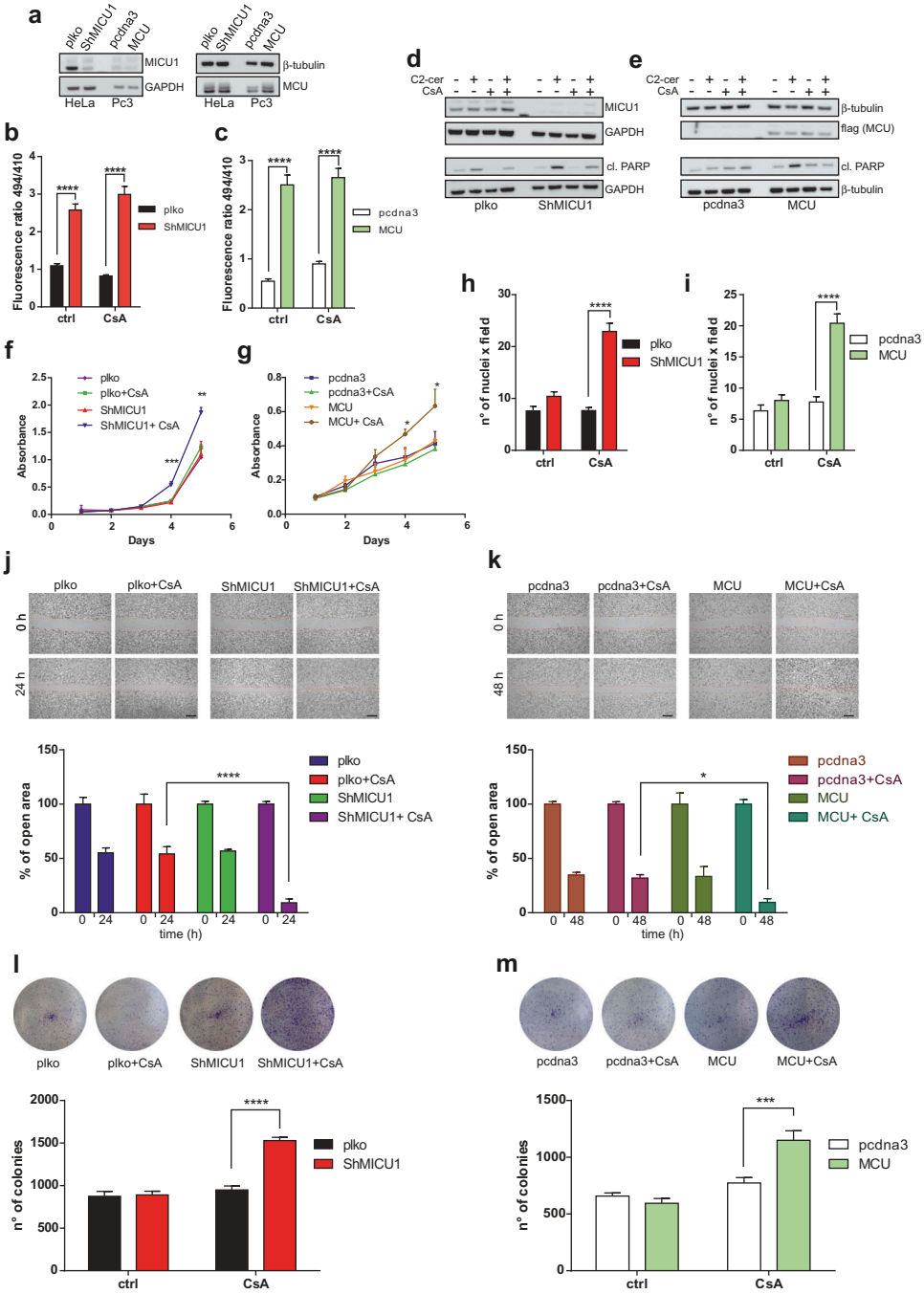

Figure 1. (a) Western blots of HeLa and PC3 cells to assess MICU1 and MCU levels. Antibodies used: GAPDH (#2118) and β-tubulin (#2128) by Cell Signalling; MICU1 (HPA037480) and MCU (HPA016480) by Sigma-Aldrich. (b-c) HeLa (b) and PC3 (c) cells were transiently transfected using Lipofectamine 2000 reagent (Thermo Fisher Scientific) with the mitochondrial-targeted GCaMP6m construct to assess basal mitochondrial Ca^{2+} levels at resting state. The cells were treated or not with 1μM Cyclosporine A (CsA). N = 4; **** $p < 0.0001$. Two-way ANOVA. (d–e) Western blots of HeLa (d) or PC3 (e) cells, treated, where indicated, with 40μM C2-ceramide (C2-cer) for 4 h or 1 μM CsA for 4 h (alone or added 1 h before C2-cer). Other antibodies used: cleaved PARP (#9541) by Cell Signalling; flag (F7425) by Sigma-Aldrich. (f–g) 10,000 HeLa (f) or PC3 (g) cells were plated in five sets of five wells of a 12-well plate. Starting from the following day (day 1), cells were treated as indicated, and 1 set of wells was washed once with PBS, fixed in 4% formaldehyde (PFA) solution for 10 min at RT, and then kept in PBS at 4°C. At day 5, all the wells were stained with crystal violet. After lysis with 10% acetic acid, the absorbance was read at 595 nm. N = 3; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Multiple *t*-test. (h–i) 1.5×10^5 HeLa (H) or 2.5×10^5 PC3 cells (I) were resuspended in medium without serum, with or without CsA, and then seeded on Transwell 8 μm pore size (Corning Incorporated 3422), using 20% FBS as attractant. After 24 h or 48 h, respectively, cells were fixed with PFA and stained with DAPI. N = 3; ****$p < 0.0001$. Two-way ANOVA. Scale bar: 200 μm. (j-k) HeLa (j) and PC3 (k) cells were grown in 6-well plates to 80–90% confluence in medium supplemented with 10% FBS. The cell monolayer was then scratched with a P200 tip, and then treated or not with CsA. Cells were allowed to close the wound for 24 h or 48 h, respectively. Migration distance was measured using the ImageJ software. N = 3; *$p < 0.05$; ****$p < 0.0001$. Two-way ANOVA. (l–m) 3000 HeLa (l) or 6000 PC3 (m) cells grown in 6-well plates, treated or not with CsA. After 10 or 15 days, respectively, cells were fixed with PFA and stained with crystal violet. Colony number was calculated using the ImageJ software. N = 3; ***$p < 0.001$; ****$p < 0.0001$; Two-way ANOVA.

cells that express MCU at higher extent (% of open area after 48 h from the wound: pcdna3+ CsA: 31.93 ± 3.43; MCU+CsA: 9.59 ± 3.64). Finally, we measured the ability to form colonies in vitro, a marker of tumorigenesis. CsA increased the number of colonies of both HeLa cells stably transduced with MICU1-directed shRNA (Figure 1(l)) and PC3 stably expressing MCU (Figure 1(m)), highlighting the pro-cancerous effects of CsA when tumor cell mitochondria can uptake Ca^{2+} at a higher degree as a consequence of aberrations in the uniporter complex composition.

In conclusion, although elevated mitochondrial Ca^{2+} entry generally predisposes to cell death, it could potentiate tumor cell proliferation and invasion when mPTP properties appear altered, a condition that frequently occurs in multiple cancer scenarios, either through post-translational events or the inhibitory activity of multiple pro-malignant factors. In this light, it has been recently shown as chronic mitochondrial Ca^{2+} aberrations, obtained through MCU depletion, could generate adaptive modifications of mPTP functions, suggesting an altered death response in cells with deregulated mitochondrial Ca^{2+} homeostasis [8]. Thus, a therapeutic strategy based on increasing mitochondrial Ca^{2+} uptake could be inefficient in cancer cells where the opening of mPTP is abolished. Conversely, blocking the pro-cancerous effects of mitochondrial Ca^{2+} entry could result in a significant reduction of tumor progression. The identification of the molecular signaling pathways that are regulated by mitochondrial Ca^{2+}, such as ATP or Radical Oxygen Species (ROS) production, as well as metabolic changes induced by mPTP inhibition, will furnish additional steps for the comprehension of the role of mitochondrial Ca^{2+} and MCU complex in cancerogenesis.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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