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Mitochondrial calcium uniporter silencing potentiates caspase-independent cell death in MDA-MB-231 breast cancer cells

Merril C. Curry [a], Amelia A. Peters [a], Paraic A. Kenny [b], Sarah J. Roberts-Thomson [a], Gregory R. Monteith [a,*]

[a] School of Pharmacy, The University of Queensland, Brisbane, Queensland 4072, Australia
[b] Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461, United States

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ABSTRACT

The mitochondrial calcium uniporter (MCU) transports free ionic Ca^{2+} into the mitochondrial matrix. We assessed MCU expression in clinical breast cancer samples using microarray analysis and the consequences of MCU silencing in a breast cancer cell line. Our results indicate that estrogen receptor negative and basal-like breast cancers are characterized by elevated levels of MCU. Silencing of MCU expression in the basal-like MDA-MB-231 breast cancer cell line produced no change in proliferation or cell viability. However, distinct consequences of MCU silencing were seen on cell death pathways. Caspase-dependent cell death initiated by the Bcl-2 inhibitor ABT-263 was not altered by MCU silencing; whereas caspase-independent cell death induced by the calcium ionophore ionomycin was potentiated by MCU silencing. Measurement of cytosolic Ca^{2+} levels showed that the promotion of ionomycin-induced cell death by MCU silencing occurs independently of changes in bulk cytosolic Ca^{2+} levels. This study demonstrates that MCU overexpression is a feature of some breast cancers and that MCU overexpression may offer a survival advantage against some cell death pathways. MCU inhibitors may be a strategy to increase the effectiveness of therapies that act through the induction of caspase-independent cell death pathways in estrogen receptor negative and basal-like breast cancers.

1. Introduction

Mitochondria regulate numerous cellular processes and are vital for both sustaining cell survival and the initiation of cell death [1,2]. The uptake of Ca^{2+} by mitochondria can buffer increases in cytosolic free Ca^{2+} ($[Ca^{2+}]_{CYT}$) and stimulate specific mitochondrial functions such as ATP synthesis [1,2]. Mitochondrial Ca^{2+} levels may also influence sensitivity to cell death activators [3,4]. In malignant transformation, remodeling of Ca^{2+} homeostasis and reprogramming of mitochondrial functions could confer cancer cells with a survival advantage and an ability to evade cell death [5,6]. Calcium transporters including the voltage-dependent anion-selective channel (VDAC) and the mitochondrial Ca^{2+} uniporter (MCU) [1] participate in mitochondrial Ca^{2+} uptake and have been proposed as potential regulators of cell death [7,8].

Since the molecular identification of MCU [9,10], several studies have investigated the significance of mitochondrial Ca^{2+} uptake on specific cellular processes through the modulation of MCU expression levels. In cardiomyocytes MCU silencing amplifies bulk $[Ca^{2+}]_{CYT}$ and is associated with increased contractile responses [11]. In breast cancer cells sustained increases of bulk $[Ca^{2+}]_{CYT}$ are associated with the promotion of cell death responses [12]. However, the potential for MCU to modulate cell death and other events in breast cancer cells has not been investigated.

MCU has been studied in other cancer types. One recent study identified MCU down-regulation as a characteristic feature of some human colon and prostate cancer cells [13]. Down-regulation of MCU in colon and prostate-derived cancers promotes increased proliferation and bestows resistance to cell death stimuli through diminished mitochondrial Ca^{2+} levels [13]. No studies have yet assessed MCU in the context of breast cancer.

In this study we assessed MCU expression in clinical breast cancers and evaluated the functional significance of MCU silencing on proliferation, cell death pathways and on bulk $[Ca^{2+}]_{CYT}$ in MDA-MB-231 breast cancer cells. Our results suggest that MCU inhibition may sensitize some breast cancers to some inducers of cell death.

Abbreviations: Ca^{2+}, calcium; $[Ca^{2+}]_{CYT}$, cytoplasmic free calcium; VDAC, voltage-dependent anion-selective channel; MCU, mitochondrial calcium uniporter; Bcl-2, B-cell lymphoma-2; siMCU, mitochondrial calcium uniporter siRNA; siNT, non-targeting siRNA; PI+, propidium iodide positive; ER, estrogen receptor; CPA, cyclopiazonic acid; ANOVA, analysis of variance; MAM, mitochondrial-associated-membrane.

* Corresponding author. Fax: +61 73346 1999.
E-mail address: gregm@uq.edu.au (G.R. Monteith).

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2. Materials and methods

2.1. Analysis of MCU levels in human breast cancer cases

Gene expression data for 180 human breast cancer cases [14] were obtained from NCBI-GEO (Accession GSE3165) and imported into Partek Genomics Suite (version 6.6). MCU (annotated on these microarrays as C10orf42) expression was analyzed in samples grouped by both estrogen receptors status and PAM50 molecular subtype [15].

2.2. Cell culture

Human MDA-MB-231 breast cancer cells (American Type Culture Collection) were grown in high glucose DMEM (Sigma Aldrich) supplemented with 10% FBS and 4 mM L-glutamine (Invitrogen) at 37 °C/5% CO_2 in a humidified air incubator.

2.3. Silencing of MCU expression

MDA-MB-231 cells were transfected as previously described [12] with ON-TARGET*plus*™ SMARTpool siRNAs (Dharmacon), consisting of four pooled siRNA sequences rationally designed to minimize off-target effects [16,17]. The specific siRNAs used in this study were MCU siRNA (siMCU, L-015519-02) and the non-targeting control siRNA (siNT, D-001810-10). For all experiments MCU mRNA silencing (>70%) was confirmed at 48 h post-siRNA.

2.4. Quantitative real time RT-PCR

Quantitative real time RT-PCR was performed as described previously [12]. Briefly, at 48 or 120 h post siRNA-transfection total RNA was isolated (RNeasy Plus mini kit; Qiagen), and then reverse transcribed (Omniscript RT kit; Qiagen). The cDNA of interest were amplified using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression assays (MCU, Hs00293548_m1; 18 S rRNA, 4319413E; Applied Biosystems) under universal cycling conditions with a StepOnePlus real time PCR system (Applied Biosystems). MCU mRNA levels were quantified by the comparative *Ct* method as described [18] normalized to 18 S rRNA and presented relative to the siNT control.

2.5. Cell enumeration and S-phase analysis

MDA-MB-231 cells were transfected with siRNA for 120 h and then cell enumeration and S-phase analysis were performed as previously described [19]. Briefly, cells with newly synthesized DNA were stained, according to the manufacturer's instructions using the Click-iT® EdU cell proliferation assay (Alexa Fluor 555; Invitrogen). DAPI-stained cell nuclei (400 nM; 90 min) and EdU-positive cells were imaged with a 10×objective using an ImageXpress Micro automated epifluorescence microscope (Molecular Devices Corporation) [20]. Cell number and percentage of EdU positive cells were calculated using the multi-wavelength cell scoring application module (MetaXpress v3.1.0.83; Molecular Devices).

2.6. Assessment of cell viability

At 72 h post siRNA-transfection MDA-MB-231 cells were treated with the cell death activators ABT-263 (Selleckchem), ionomycin (Enzo Life Sciences), or with dimethyl sulfoxide (up to 0.33%) and incubated for an additional 48 h. Cell viability was then assessed in non-fixed cells as previously reported by evaluating Hoechst 33342 (10 μg/mL; Invitrogen) and propidium iodide (1 μg/mL; Invitrogen) staining [12]. Imaging was performed using an ImageXpress Micro automated epifluorescence microscope (Molecular Devices Corporation). Criteria for viable and propidium iodide positive cells (PI+) were defined as previously described [12].

2.7. Cytosolic free Ca^{2+} measurements

At 72 h post siRNA-transfection MDA-MB-231 breast cancer cells were loaded with either a high Ca^{2+} affinity (Fluo-4AM (4 μM; Molecular Probes)) or a low Ca^{2+} affinity (Fluo-4FF (4 μM; Molecular Probes)) Ca^{2+} indicator according to published methods [12]. $[Ca^{2+}]_{CYT}$ was then monitored using a fluorescence imaging plate reader [21] (Molecular Devices Corporation) as previously described [12]. To assess relative $[Ca^{2+}]_{CYT}$ fluorescence was normalized to base-line values.

2.8. Statistical analysis

Statistical tests were performed as described in the figure legends using GraphPad Prism version 5.04 for Windows.

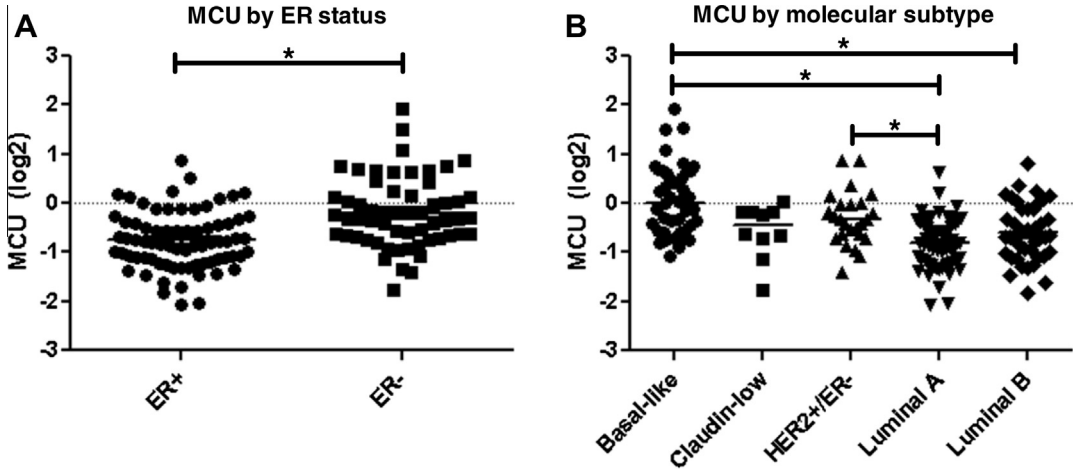

Fig. 1. MCU expression in clinical breast cancers is associated with estrogen receptor status and molecular subtype. Relative MCU levels were analyzed in human breast cancer cases (n = 180) stratified by (A) estrogen receptor (ER) status and (B) molecular subtype. MCU levels were highest in ER-negative tumors ($P < 0.05$, Mann–Whitney test) and showed the strongest enrichment in the basal-like subtype ($P < 0.05$; basal-like versus Luminal A and B, Kruskall–Wallis test with Dunn's post-test for multiple comparisons).

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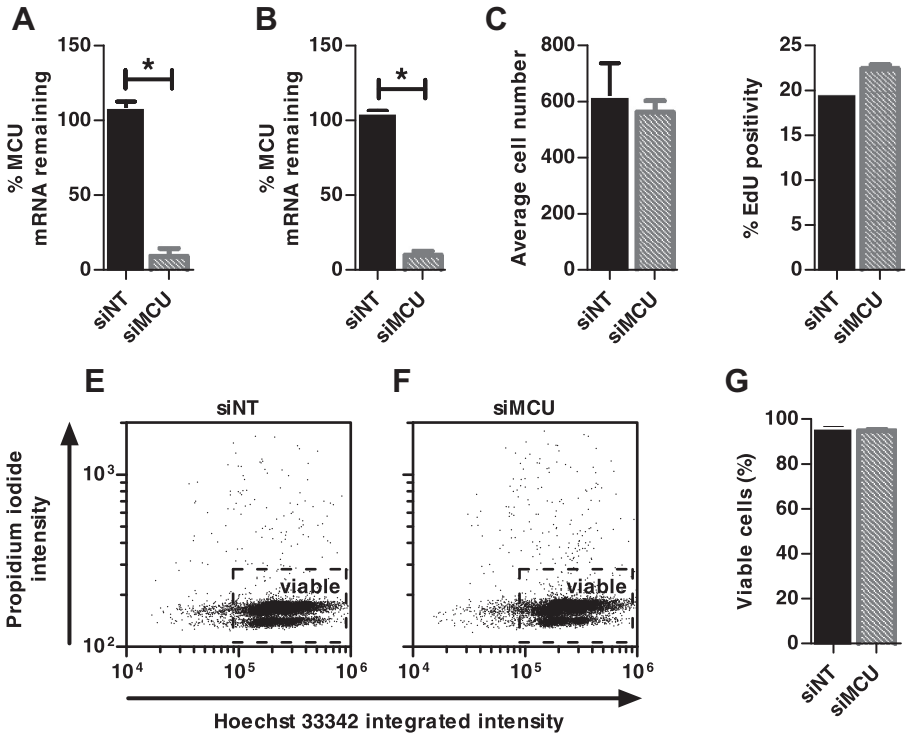

Fig. 2. MCU silencing in MDA-MB-231 cells and the effects of silencing on proliferation and cell viability. MCU was silenced in MDA-MB-231 cells using siRNA and the effects on cell number, S-phase and cell viability were compared to the siNT control. Relative MCU mRNA levels at (A) 48 h and (B) 120 h post-transfection with siMCU or siNT. Effects of MCU knockdown on (C) average cell number, (D) percentage S-phase (EdU-positive cells) and (G) percentage viable cells. Bar graphs show the mean ± S.D obtained from three independent experiments ($n = 3$) performed in triplicate. Dot plots show changes in cell viability with either (E) siNT or (F) siMCU for 10,000 cells randomly selected from three independent experiments ($n = 3$) performed in triplicate wells. $^{*}P < 0.05$, paired two-tailed t test.

3. Results

3.1. MCU mRNA levels in clinical breast cancer samples

Assessment of MCU mRNA levels in a cohort of 180 breast cancer cases [14] showed a significantly ($P < 0.05$; Fig. 1A) higher level of MCU in estrogen receptor (ER)-negative breast cancers. When breast cancers were stratified by molecular subtype the highest levels of MCU were seen in basal-like breast cancers ($P < 0.05$; basal-like versus luminal A and B subtypes; Fig. 1B), while other subtypes expressed intermediated MCU levels (Fig. 1B). ER-negative and basal-like breast cancers are associated with a particularly poor prognosis [22,23].

3.2. Effects of MCU silencing on proliferation and cell viability in MDA-MB-231 breast cancer cells

To assess the possible role of MCU overexpression in breast cancer, we used specific siRNAs to silence MCU expression in basal-like MDA-MB-231 breast cancer cells [24]. Compared to the siNT control, cells transfected with siMCU showed a significant ($P < 0.05$) decrease in MCU mRNA levels at 48 h (Fig. 2A) and 120 h (Fig. 2B) post-siRNA. Using this model we assessed the effects of MCU knockdown on proliferation and cell viability.

Effects of MCU silencing on cell proliferation were evaluated by cell enumeration and S-phase analysis (EdU staining). MCU silencing did not significantly alter cell number (Fig. 2C), or the percentage of cells in S-phase (EdU positive; Fig. 2D) compared to the siNT control. Cell viability was also quantified; MCU silencing had no significant effect on the percentage of viable cells compared to the siNT control (Fig. 2E–G).

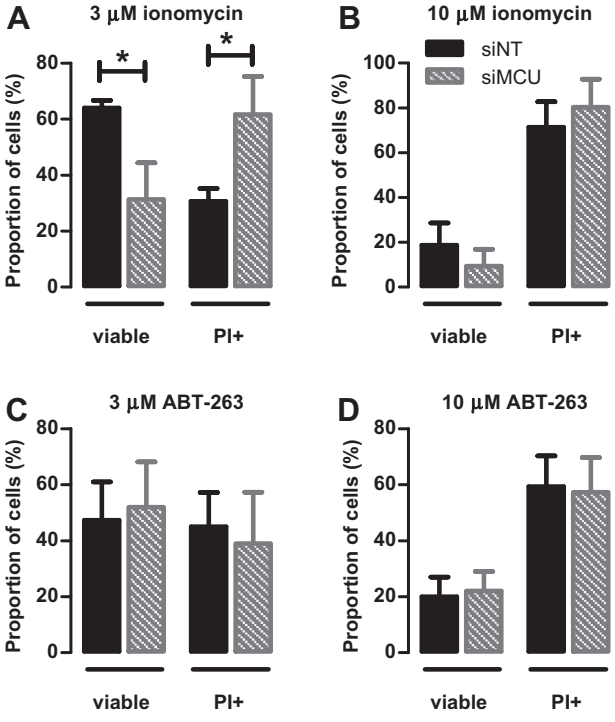

Fig. 3. Consequences of MCU silencing on ionomycin- and ABT-263-initiated cell death in MDA-MB-231 cells. MCU was silenced in MDA-MB-231 cells using siRNA and then cells were treated with (A) 3 µM ionomycin, (B) 10 µM ionomycin, (C) 3 µM ABT-263 or with (D) 10 µM ABT-263 for 48 h prior to cell death assessment. Bar graphs show mean ± SD of the proportion of viable and propidium iodide positive (PI+) cells in siMCU and siNT transfected cells. Data were obtained from three independent experiments ($n = 3$) performed in triplicate. $^{*}P < 0.05$, repeated measures two-way ANOVA, Bonferroni post hoc analysis.

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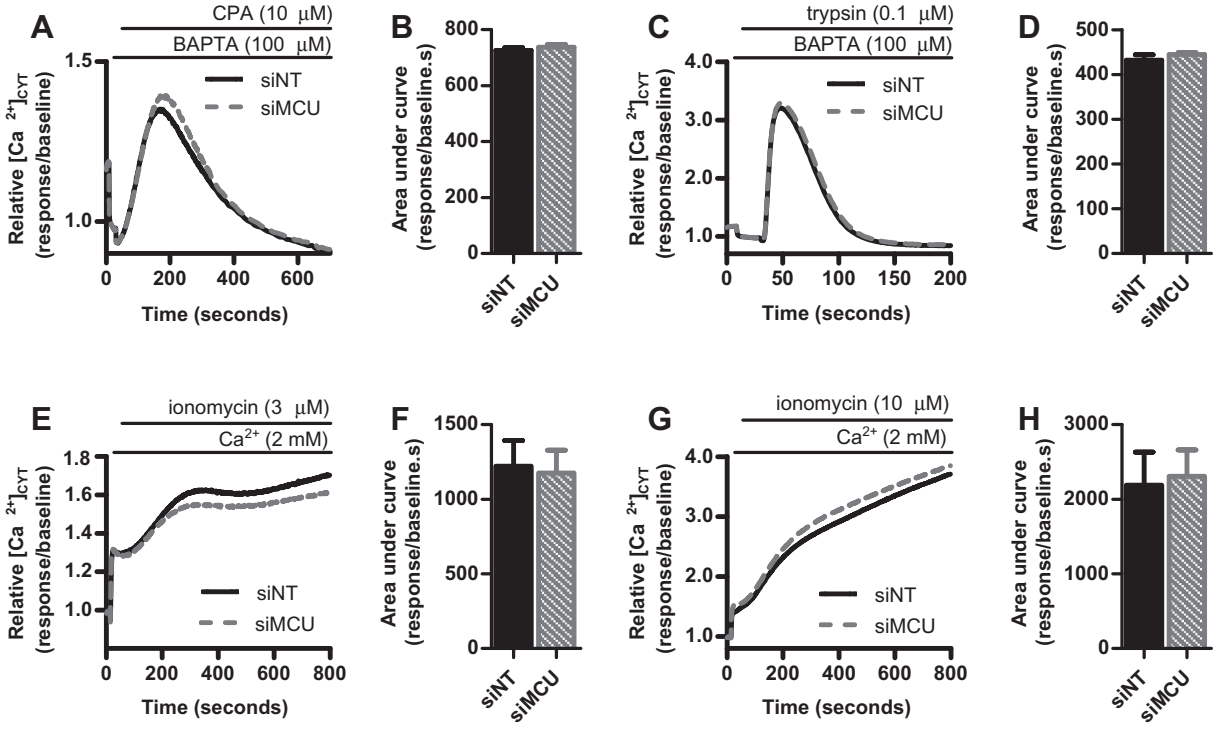

Fig. 4. Effect of MCU silencing on bulk $[Ca^{2+}]_{CYT}$ increases stimulated by various initiators of $[Ca^{2+}]_{CYT}$ increases. (A) CPA (10 μM)-mediated and (C) trypsin (0.1 μM)-induced $[Ca^{2+}]_{CYT}$ transients measured with the Ca^{2+} indicator Fluo-4AM after transfection with siMCU or siNT in the presence of extracellular BAPTA (100 μM). (E) 3 μM ionomycin and (G) 10 μM ionomycin $[Ca^{2+}]_{CYT}$ transients recorded using the Ca^{2+} indicator Fluo-4FF in cells transfected with siNT or siMCU. Bar graphs show the mean ± S.D for the area under the curve calculated for the (B) 10 μM CPA, (D) 0.1 μM trypsin, (F) 3 μM ionomycin and (H) 10 μM ionomycin $[Ca^{2+}]_{CYT}$ increases. Ca^{2+} traces represent relative mean fluorescence. All data were obtained from three independent experiments ($n = 3$) performed in triplicate. $^*P < 0.05$, paired two-tailed t test.

3.3. MCU silencing sensitizes MDA-MB-231 breast cancer cells to ionomycin-mediated cell death

To analyze whether MCU silencing regulates breast cancer cell responses to death activators, siRNA-transfected MDA-MB-231 cells were treated with the Ca^{2+} ionophore ionomycin to initiate caspase-independent cell death [12]. Compared to the siNT control MCU silencing potentiated sub-maximal ionomycin (3 μM)-mediated cell death, with a significant ($P < 0.05$) reduction in cell viability and a marked ($P < 0.05$) increase in the proportion of propidium iodide positive cells (Fig. 3A). Silencing of MCU did not alter cell viability at a higher ionomycin concentration (10 μM) compared to the siNT control (Fig. 3B). These data suggest MCU silencing sensitizes MDA-MB-231 cells to caspase-independent cell death initiated with ionomycin.

3.4. MCU silencing does not modulate ABT-263-mediated MDA-MB-231 breast cancer cell death

The Bcl-2 inhibitor ABT-263 was used to address the consequence of MCU silencing on caspase-dependent cell death in the MDA-MB-231 breast cancer cell line [12]. Compared with the siNT control, MCU silencing exerted no effect on cell death initiated at either the submaximal (3 μM; Fig. 3C) or high (10 μM; Fig. 3D) ABT-263 concentrations. This suggests that MCU knockdown does not sensitize MDA-MB-231 cells to caspase-dependent cell death initiated by ABT-263.

3.5. Effects of MCU silencing on increases in bulk $[Ca^{2+}]_{CYT}$

To assess whether MCU silencing modified the nature of bulk $[Ca^{2+}]_{CYT}$ increases, we challenged siRNA-transfected MDA-MB-231 breast cancer cells with different initiators of $[Ca^{2+}]_{CYT}$ increases. Moderate increases in bulk $[Ca^{2+}]_{CYT}$ were induced with the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA; 10 μM) or the protease activated receptor activator trypsin (0.1 μM) under Ca^{2+}-free conditions. Compared with the siNT control, silencing of MCU did not alter either CPA (Fig. 4A) or trypsin (Fig. 4C) Ca^{2+} responses, with no significant difference detected for area under the curve (Fig. 4B and D) or other Ca^{2+} transient parameters such as peak $[Ca^{2+}]_{CYT}$ and half peak decay time (data not shown).

A role for MCU in shaping Ca^{2+} signals of higher magnitude was evaluated in the presence of extracellular Ca^{2+} (2 mM) using the Ca^{2+} ionophore ionomycin. Assessment of $[Ca^{2+}]_{CYT}$ with the low affinity Ca^{2+} indicator Fluo-4FF, showed that MCU silencing compared to the siNT control had no major effects on bulk $[Ca^{2+}]_{CYT}$ increases, at either submaximal (3 μM; Fig. 4E and F) or high (10 μM; Fig. 4G and H) ionomycin concentrations. Collectively, these findings demonstrate that MCU does not have a major role in the regulation of bulk $[Ca^{2+}]_{CYT}$ in MDA-MB-231 breast cancer cells.

4. Discussion

A previous study identified MCU downregulation in prostate and colon-derived cancers [13], here we present data showing that MCU is upregulated in some clinical breast cancer subtypes. MCU was most highly expressed in ER-negative and basal-like breast cancers both of which are associated with a poor prognosis [22,23]. To understand the implication of MCU overexpression in breast cancer, we assessed the functional consequences of MCU silencing on proliferation, cell death and bulk $[Ca^{2+}]_{CYT}$ in the basal-like MDA-MB-231 breast cancer cell line.

Our silencing studies in MDA-MB-231 cells showed MCU knockdown had no effect on proliferation or cell viability.

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However, assessment of MCU knockdown on cell death initiated by ABT-263 and ionomycin defined a distinct role for MCU in the regulation of caspase-independent and -dependent cell death pathways, respectively. MCU silencing did not affect ABT-263-mediated cell death, but acted as a sensitizer of cell death initiated with ionomycin. MCU silencing attenuates mitochondrial Ca^{2+} uptake [9,10] and reduced mitochondrial Ca^{2+} levels act to suppress cell death in a variety of malignant cell lines, including those derived from the cervix [4] central nervous system [25], head and neck [26] and lymph [27] tissues. We therefore hypothesized that MCU knockdown in MDA-MB-231 breast cancer cells would also protect from death insults. However, MCU knockdown did not protect against ABT-263-induced cell death, and potentiated ionomycin-initiated cell death.

Alterations in bulk $[Ca^{2+}]_{CYT}$ signals were examined as a potential mechanism of MCU silencing-mediated promotion of ionomycin-initiated cell death. In cardiomyocytes MCU silencing amplifies cytosolic Ca^{2+} signals [11] and we have recently shown sustained increases of bulk $[Ca^{2+}]_{CYT}$ are associated with the promotion of ionomycin-mediated MDA-MB-231 cell death [12]. However, our assessment of bulk $[Ca^{2+}]_{CYT}$ showed no major changes upon MCU knockdown on Ca^{2+} signals stimulated with CPA, trypsin or ionomycin. These data suggest that MCU does not significantly contribute in buffering global cytosolic free Ca^{2+}; therefore increases in bulk $[Ca^{2+}]_{CYT}$ are unlikely to explain the promotion of ionomycin-initiated cell death upon MCU silencing.

The ability of MCU silencing to sensitize MDA-MB-231 cells to ionomycin-mediated cell death could be due to diminished mitochondrial Ca^{2+} uptake and alterations in the autophagy survival pathway, as the specific MCU inhibitor Ru360 regulates autophagy in HEK293 and DT40 cells [28]. Another mechanism may involve alterations in localized Ca^{2+} signals within specialized signaling domains at the endoplasmic reticulum-mitochondrial interface, known as mitochondrial-associated-membranes (MAMs) [29]. A recent study investigated reduced mitochondrial Ca^{2+} uptake through knockdown of the MAM-enriched calcium transporter, VDAC1 [30]. VDAC1 silencing potentiates the response of human-derived HeLa cervical cancer cells to death activators without affecting bulk $[Ca^{2+}]_{CYT}$, this sensitization was not dependent on diminished mitochondrial Ca^{2+} levels [30]. Localized Ca^{2+} signals possibly within MAM domains may represent another regulatory site for Ca^{2+}-dependent death signals in breast cancer cells and along with MCU-mediated regulation of autophagy requires further investigation.

In this report, we have identified MCU overexpression in some clinical breast cancers, in particular those subtypes associated with a poor prognosis (estrogen receptor negative and basal-like). MCU silencing in the basal-like MDA-MB-231 breast cancer cell line promoted ionomycin-initiated but not ABT-263-mediated cell death. MCU inhibitors may therefore represent novel therapies for some breast cancers by potentiating some caspase-independent cell death mechanisms.

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