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

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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.

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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.

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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.

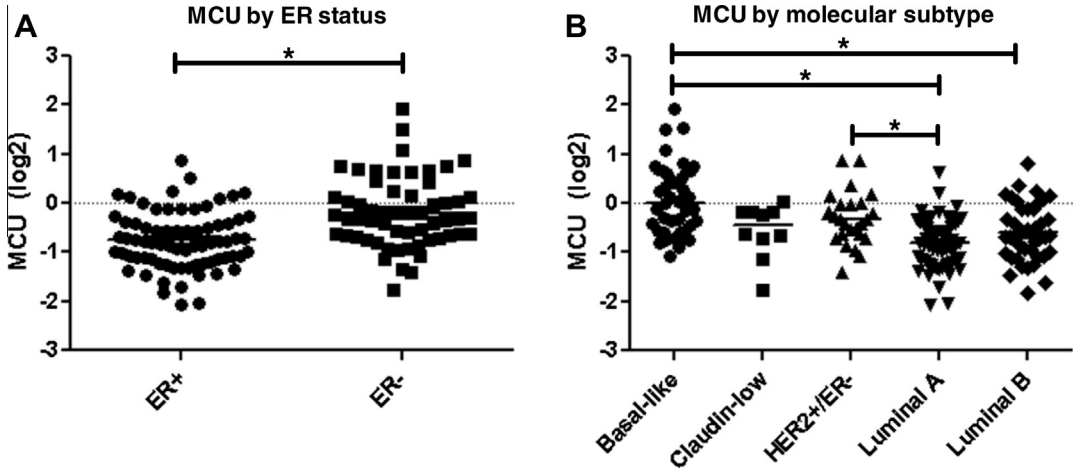

Fig. 1. MCU expression in clinical breast cancers is associated with estrogen receptor status and molecular subtype. Relative MCU levels were analyzed in human breast cancer cases (n = 180) stratified by (A) estrogen receptor (ER) status and (B) molecular subtype. MCU levels were highest in ER-negative 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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