Downregulation of the Mitochondrial Calcium Uniporter by Cancer-Related Calcium miR-25

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Summary

The recently discovered mitochondrial calcium uniporter (MCU) promotes calcium accumulation into the mitochondrial matrix[1, 2]. We identified in silico miR-25 as a cancer-related MCU-targeting microRNA family and demonstrate that its overexpression in HeLa cells drastically reduces MCU levels and mitochondrial calcium uptake, while leaving other mitochondrial parameters and cytosolic calcium signals unaffected. In human colon cancers and cancer-derived cells, miR-25 is overexpressed and MCU accordingly silenced. miR-25-dependent reduction of mitochondrial calcium uptake correlates with resistance to apoptotic challenges and can be reversed by anti-miR-25 overexpression. Overall, the data demonstrate that microRNA targeting of mitochondrial calcium signaling favors cancer cell survival, thus providing mechanistic insight into the role of mitochondria in tumorigenesis and identifying a novel therapeutic target in neoplasia.

Results and Discussion

miR-25 Downregulates MCU and Protects from Calcium-Dependent Apoptosis

In the last two decades, mitochondrial calcium homeostasis has been shown to participate in the control of the intrinsic pathway of apoptosis and to be influenced by oncoproteins[3–6], thus suggesting that it is a signaling checkpoint in tumorigenesis. However, direct evidence and mechanistic insight were still lacking. The recent identification of the mitochondrial calcium uniporter, MCU[1, 2] and of the associated regulator MICU1 (also known as CBARA1)[7] now allow molecular investigation of the process, including the regulation of their expression by microRNAs (miRNAs). miRNAs are a class of small (19–25 nt), noncoding regulatory RNAs that regulate gene expression, causing target mRNA degradation or suppressing mRNA translation[8]. In human cancers, specific miRNAs are up- or downregulated, with consequent alteration in the expression of target proteins[9, 10]. By filtering the output of four target prediction algorithms (TargetScan[11], MicroT[12], MicroCosm[13], and miRanda[14]; see Table S1 available online), we identified five cancer-related miRNA families (miR-15, miR-17, miR-21, miR-25, and miR-137) that could be predicted to target MCU and/or MICU1. We thus tested their effect on mitochondrial calcium homeostasis by expressing them in HeLa cells and measuring mitochondrial calcium homeostasis by observing them in HeLa cells and measuring mitochondrial calcium.[25] with a targeted aequorin-based calcium probe (mtEIQ) [15]. The data (Figure 1A) showed that only miR-25 caused a marked reduction in the calcium rise evoked by cell stimulation with 100 μM histamine, an agonist coupled to the generation of inositol 1,4,5-trisphosphate (InsP₃) and the release of calcium from the endoplasmatic reticulum (ER). Accordingly, overexpression of an anti-miR-25 increases mitochondrial calcium uptake to agonist stimulation (Figure S1A), with a slight decrease in cytosolic calcium (Ca²⁺) (Ca²⁺), probably due to increased calcium clearance by mitochondria (Figure S1B).

The effects were predicted to depend on MCU downregulation. Indeed, the bioinformatics analysis of the 1,896 nt 3' UTR of MCU revealed a 100% match target seed sequence for miR-25 at nt 1075–1081, highly conserved across seven species (Figure 1B), and insertion of the 759 nt 3' UTR of MCU (but not of the 569 nt 3' UTR of MICU1) downstream of the luciferase gene in a reporter plasmid led to significant miR-25-dependent decrease of reporter activity (Figures S1C and S1D). We thus tested MCU expression by immunoblotting and detected a marked reduction in the protein level upon miR-25 overexpression (Figure 1C) and an increase in anti-miR-25-expressing cells (Figure S1E). As expected, MCU mRNA abundance was significantly decreased by miR-25 (Figure 1D), whereas anti-miR-25 increased it (Figure S1F). MCU downregulation was also evident using an immunofluorescence technique: Figure S1G shows that miR-25 expression drastically decreased MCU antibody reactivity.

The effect of miR-25 is shared by the other members of the miRNA family: miR-92a and miR-363 target MCU mRNA and reduce MCU protein levels and, accordingly, inhibit mitochondrial calcium uptake, without affecting calcium (Ca²⁺) and calcium (Ca²⁺) (data not shown).

We investigated whether miR-25-dependent reduction in mitochondrial calcium uptake correlates with increased resistance to apoptotic challenges. Microscopy counts of cell viability after treatment with H₂O₂, C₂-ceramide, or staurosporine (STS) revealed that miR-25-expressing HeLa cells were strongly protected from death caused by C₂-ceramide and H₂O₂ (Figure 1E), apoptotic challenges for which mitochondrial calcium loading acts as a sensitizing factor[16–18], whereas the sensitivity to STS was unaffected. Accordingly, PARP and caspase-3 cleavage upon C₂-ceramide treatment were markedly reduced in miR-overexpressing cells (Figure 1F). These results were also confirmed by cellular positivity to the apoptotic marker annexin V (Figure S1H).
miR-25 Induces Reduction of Mitochondrial Ca\(^{2+}\) Uptake Exclusively through MCU

We then proceeded to rule out that the effect on \([\text{Ca}^{2+}]_{\text{m}}\) was secondary to alterations of global Ca\(^{2+}\) signaling patterns or to morphological or functional dysregulation of mitochondria. On the former aspect, we investigated the cytosolic \([\text{Ca}^{2+}]_{\text{c}}\) changes and the state of filling and release kinetics of the ER. The results showed that miR-25, when expressed in HeLa cells, caused no difference in the amplitude of the \([\text{Ca}^{2+}]_{\text{c}}\) rise evoked by histamine (Figure 2A), nor in the steady state \([\text{Ca}^{2+}]_{\text{er}}\) or in the release caused by the agonist (Figure 2B). Thus, the effect of miR-25 on Ca\(^{2+}\) homeostasis is exclusively mitochondrial.

We then investigated the mitochondrial membrane potential (\(\Delta\Psi_m\)), the driving force for Ca\(^{2+}\) accumulation, and the morphology of mitochondria, i.e., both the contacts with the ER (which were shown to be a critical determinant of rapid Ca\(^{2+}\) transfer between the two organelle [19–21]) and the formation of largely interconnected tubules, which favors Ca\(^{2+}\) diffusion within mitochondria. On the former aspect, measurements with the \(\Delta\Psi_m\)-sensitive fluorescent dye tetramethylrhodamine methyl ester (TMRM) revealed no difference between miR-overexpressing and control HeLa cells (Figure 2C). As to morphology, mitochondrial labeling with the fluorescent probe mtDsRed showed that miR-25 overexpression causes no significant difference in mitochondrial volume or number (Figure 2D). Similarly, cotransfection with mtDsRed and an ER-targeted GFP showed no difference in the number of contact sites (Figure 2D, contact sites in white).

Overall, the data reveal that the \([\text{Ca}^{2+}]_{\text{m}}\) reduction caused by miR-25 should be ascribed to reduction of mitochondrial Ca\(^{2+}\) uptake through MCU. To further confirm this notion, we measured mitochondrial Ca\(^{2+}\) accumulation in permeabilized cells. For this purpose, HeLa cells were perfused with a solution mimicking the intracellular milieu (IB), supplemented with 2 mM EGTA, and permeabilized with digitonin for 1 min. The perfusion buffer was then changed to IB with an EGTA-buffered \([\text{Ca}^{2+}]_{\text{o}}\) of 4 \(\mu\)M (Figure 2E) or 1 \(\mu\)M (Figure 2F), eliciting a gradual rise in \([\text{Ca}^{2+}]_{\text{m}}\) that reached a plateau value of \(\sim 80\) and \(\sim 20\), respectively. At both buffered \([\text{Ca}^{2+}]_{\text{o}}\), miR-25
overexpression causes a marked reduction in the rate of Ca^{2+} accumulation into mitochondria.

Mitochondrial Ca^{2+} alterations induced by miR-25 could be reverted by MCU re-expression in miR-25-expressing cells (Figure S2A) and, accordingly, this rescued Ca^{2+} affinity was mirrored in enhanced susceptibility to Ca^{2+}-dependent apoptosis (Figure S2B). Moreover, 22Rv1 prostatic cells, which possess very high levels of miR-25 (see Figure 3), were strongly sensitized to apoptosis after MCU overexpression (Figure S2 C). The increased ability of mitochondria to accumulate Ca^{2+} is a fundamental aspect in MCU-related promotion of cell death: indeed, apoptosis induction observed in MCU-overexpressing HeLa cells was almost abolished in the presence of intracellular Ca^{2+} buffer BAPTA (Figure S2 D).

Finally, although miR-25 has also been reported to exert antiapoptotic effects via interference with the expression of proapoptotic proteins, such as Bim [22], TRAIL [23], and PTEN [24], these results show how MCU can be considered a fundamental target of miR-25-dependent apoptosis inhibition.

**Inhibition of MCU Levels by miR-25 Is a Key Aspect in Human Colon Cancer Progression**

We then extended the analysis to cancer cells and tissues. We first evaluated cell lines derived from human carcinomas, in which miR-25 was reported to be highly expressed [24–26]. Both in PC3, LnCaP, and 22Rv1 (derived from prostate cancer) and in HCT116, RKO, SW80, and WiDr (derived from colon cancer) cell lines, we detected an inverse correlation between miR-25 levels and MCU mRNA expression, with high miR-25 levels and low MCU expression levels in cancer lines, compared to primary nonneoplastic cells (Figure 3 A). We then directly investigated human poorly differentiated colonic adenocarcinoma samples by immunohistochemistry and microarray. Also in this case, a significant difference in miR-25 expression levels was detected (Figure 3 B), which correlates with a downregulation of MCU expression. Indeed, in colonic adenocarcinoma samples with high miR-25 expression levels, MCU was virtually undetectable by immunohistochemistry in cancerous tissues, compared to relatively high protein abundance in the normal mucosa (Figure 3 C).

To validate that miR-25 exerts its biological activity through its effect on MCU, we transfected HeLa cells with short hairpin RNA (shRNA) targeting MCU: as for miR-25, shRNA-MCU decreases MCU abundance and increases proliferation (Figure S3 A), indicating that MCU targeting is important for the growth-promoting activity of miR-25. We also tested the ability of MCU to inhibit the proliferation. We generated PC3 cells that stably expressed a MCU-FLAG-tagged construct (MCU-FLAG), in which MCU level and activity was increased relative to that in empty vector (pcDNA3) stable clones (Figures S3 B and S3C), and found that they formed lower numbers of colonies in soft agar compared to control pcDNA3 stable clones (Figure S3D).
mRNA and MCU expression results, respectively, for each sample. Primary nonneoplastic cells present very low abundance of miR-25 and high MCU levels, whereas cancer lines are characterized by inverse correlation between miR-25 levels and MCU mRNA expression. Error bars correspond to mean ± SEM of n = 3 independent experiments.

(B) mRNA expression was assessed in 44 normal mucosa samples and 59 stage 2–3 CRC samples via microarray. The graph shows the average expression level of miR-25 in both groups. miR-25 was significantly overexpressed in cancer samples, as compared to normal mucosa (p < 0.0001).

(C) Upper row: normal colonic mucosa (routinely stained with hematoxylin and eosin, at left) demonstrated strong cytoplasmic granular reactivity with the anti-MCU antibody (immunoperoxidase staining, at right). Lower row: poorly differentiated colonic adenocarcinoma with solid pattern of growth (hematoxylin and eosin, at left) showing low level of reactivity with the anti-MCU antibody (immunoperoxidase staining, at right). Two neoplastic cells with cytoplasmic immunoreactivity of moderate intensity can be observed. See also Figure S3.

We then investigated whether miR-25-dependent inhibition of mitochondrial Ca$^{2+}$ uptake, and the ensuing resistance to apoptosis, could be specifically reversed in cancer cells. For this purpose, we overexpressed anti-miR-25 in the PC3 and HCT116 cell lines investigated in Figure 3. In both cell types, anti-miR-25 expression caused an ~40% increase in the [Ca$^{2+}$]m rise evoked by 100 μM ATP (Figures 4A and 4B). Accordingly, sensitivity to C2-ceramide and H$_2$O$_2$ was enhanced, as revealed by the lower viability (Figures 4C and 4D) and increased PARP and caspase-3 cleavage (Figures 4E and 4F) detected in anti-miR-25-expressing cells. These data were also confirmed measuring cellular positivity to annexin V (Figures S4A and S4B).

Overall, the data identify a microRNA (miR-25), highly expressed in cancer cells, that by targeting the newly discovered calcium channel of mitochondria reduces the sensitivity of cancer cells to apoptotic agents. This not only represents conclusive evidence of the key role of organelle Ca$^{2+}$ accumulation in the mitochondria-dependent apoptotic routes but also highlights a novel, unexpected target in cancer therapy. Now, the exciting task of unveiling the structural and functional properties of this long-awaited component of the calcium signaling machinery of the cell finds an immediate translational application in a disease area of paramount importance.

Experimental Procedures

Cell Culture and Transient Transfection

HeLa, Hek293, HCT116, and RKO cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), L-glutamine, and penicillin/streptomycin in 75 cm$^2$ Falcon flasks. PC3, 22Rv1, and LnCaP cells were cultured in RPMI 1640, supplemented with 10% FCS, 2 mM L-glutamine, and penicillin/streptomycin, in 75 cm$^2$ Falcon flasks.

For aequorin experiments, cells were seeded onto 13 mm glass coverslips and allowed to grow to 75% confluence; for microscopy counts of cell viability, mitochondrial/reticular morphology analysis, and mitochondrial membrane potential measurements, cells were seeded on 24 mm glass coverslip in the same growth conditions. After 24 hr transfection with the indicated miR, cells were treated with apoptotic stimuli (H$_2$O$_2$, C2-ceramide, or staurosporine), washed three times, and fixed and imaged with a microscope. For aequorin measurements, cells were seeded onto 13 mm glass coverslips in the same growth conditions.

Aequorin Measurements

Probes employed were chimeric aequorins targeted to the endoplasmic reticulum (erAEQmut), cytosol (cytAEQ), and mitochondria (mtAEQmut). “AEQ” refers to wild-type aequorin, and “AEQmut” refers to a low-affinity D119A mutant of aequorin, as described previously (see Supplemental Experimental Procedures).

Immunoblotting

Total cell lysates were prepared in RIPA buffer, and standard immunoblotting procedures were used (Supplemental Experimental Procedures).

Apoptosis Assay

After 24 hr transfection with the indicated miR, cells were treated with apoptotic stimuli (H$_2$O$_2$, C2-ceramide, or staurosporine), washed three
times in PBS, and then fixed with 4% formaldehyde for 10 min at room temperature (RT). Cells were rinsed with PBS, and 0.1 mg/ml DAPI was added for 10 min at RT. After washing with PBS, the cells were detected with fluorescence microscopy, and cells with condensed and/or fragmented chromatin indicative of apoptosis were not counted as living cells. 250 fields per well were counted using a Scanr high-content-throughput system (Olympus).

Immunohistochemistry
Sections (4 μm thick) were cut from formalin-fixed paraffin-embedded blocks. One section for each block was routinely stained with hematoxylin and eosin for histological examination (Supplemental Experimental Procedures).

Microarray and Data Analysis
RNA labeling and hybridization on microRNA microarray chips (ArrayExpress accession number A-MEXP-258) were performed as described previously [25]. Raw data were normalized and analyzed in GeneSpring GX software version 7.3 (Silicon Genetics or Agilent Technologies). Values below 0.01 were set to 0.01. Each measurement was divided by the 50th percentile of all measurements in that sample. GeneSpring software generated a unique value for each miRNA, performing the average of four probes. Graphs and statistical analyses were performed using GraphPad Prism 5 software.

Supplemental Information
Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.11.026.

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Figure 4. Regulation of miR-25 Levels Strongly Sensitizes Cells to Ca2+-Dependent Apoptotic Stimuli
(A) [Ca2+]m peaks in PC3 cells: Ctrl miR: 5.25 ± 0.59 μM; anti-miR-25: 7.81 ± 0.64 μM. n = 16 independent experiments.
(B) [Ca2+]m peaks in HCT116 cells: Ctrl miR: 2.28 ± 0.21 μM; anti-miR-25: 3.32 ± 0.31 μM. n = 16 independent experiments.
(C and D) Microscopic counts of cell viability in PC3 (C) and HCT116 (D) cells. Treatments with H2O2 (500 μM for 2 hr) and C2-ceramide (C2-cer.; 40 μM for 2 hr) reveal a more efficient apoptosis induction after anti-miR-25 transfection. n = 3 independent experiments.
(E and F) Immunoblot shows increased levels of cleaved PARP and cleaved caspase-3 in anti-miR-25-expressing PC3 (E) and HCT116 (F) cells after treatment with C2-ceramide (C2-cer.; 40 μM for 2 hr). *p < 0.05; error bars correspond to mean ± SEM. See also Figure S4.
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