Ryanodine Receptor 1-mediated Ca^{2+} signaling and mitochondrial reprogramming modulate uterine serous cancer malignant phenotypes

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Abstract

Background

Uterine serous cancer (USC) is the most common non-endometrioid subtype of uterine cancer, and is also the most aggressive. Most patients will die of progressively chemotherapy-resistant disease, and the development of new therapies that can target USC remains a major unmet clinical need. This study sought to determine the molecular mechanism by which a novel unfavorable prognostic biomarker RYR1 identified in advanced USC confers their malignant phenotypes, and demonstrated the efficacy of targeting RYR1 by repositioned FDA-approved compounds in USC treatment.

Methods

TCGA USC dataset was analyzed to identify top genes that are associated with patient survival and can be targeted by FDA-approved compounds. The top gene RYR1 was selected and the functional role of RYR1 in USC progression was determined by silencing and over-expressing RYR1 in USC cells *in vitro* and *in vivo*. The molecular mechanism and signaling networks associated with the functional role of RYR1 in USC progression were determined by reverse phase protein arrays (RPPA), Western blot, and transcriptomic profiling analyses. The efficacy of the repositioned compound dantrolene on USC progression was determined using both *in vitro* and *in vivo* models.

Results

High expression level of ryanodine receptor 1 (RYR1) in the tumors is associated with shortened overall survival. Inhibition of RYR1 suppressed proliferation, migration and enhanced apoptosis through the Ca^{2+} -dependent AKT/CREB/PGC-1 α and AKT/HK1/2 signaling pathways, which modulate mitochondrial bioenergetics properties, including oxidative phosphorylation, ATP production, mitochondrial membrane potential, ROS production and TCA metabolites, and glycolytic activities in USC cells. Repositioned compound dantrolene suppressed USC progression in both *in vitro* and mouse models.

Conclusions

These findings provide insight into the mechanism by which RYR1 modulates the malignant phenotypes of USC and could aid in the development of dantrolene as a repurposed therapeutic agent for the treatment of USC to improve patient survival.

Statement Of Significance

Our data provides the first evidence that RYR1 upregulation is associated with USC patient survival. The high RYR1 expression plays essential roles during USC progression. We propose inhibition of RYR1 by

dantrolene as a therapeutic avenue to treat USC patients.

Background

Uterine serous cancer (USC) is the most common non-endometrioid subtype of uterine cancer. It is also the most aggressive, accounting for no more than 10% of all endometrial cancers but 40% of correlative deaths [1, 2]. In the United States, approximately 5000 new cases are diagnosed and 4000 patients die of the disease annually [3].

USC shares many similarities with high-grade serous ovarian cancer (HGSOC), which accounts for most of the 20,000 yearly new cases of epithelial ovarian cancer in the United States [4] including similar age/geographic distribution, risk factors, histopathological features and common genomic signatures such as *TP53* mutation rates [5]. USC usually sensitive to initial platinum- and taxane-based chemotherapy (>75% response rates) [6, 7]. However, most of these tumors (>75-80%) will recur within 12 to 24 months after diagnosis, and these patients will die of progressively chemotherapy-resistant disease [7]. These findings suggest that development of new therapies that can target USC remains a major unmet clinical need.

Drug repositioning, a promising field in drug discovery and development, is the process of redeveloping a compound for use in different diseases [8]. Drug repositioning has many advantages over traditional *de novo* drug discovery approaches, since it can significantly reduce the cost and time for drug development. Furthermore, repurposed or repositioned drugs have demonstrated safety in humans and therefore negate the need for phase I clinical trials [9]. However, repurposed drugs that can be used for USC treatment have not been widely explored.

The purpose of our study was to identify unfavorable prognostic biomarkers in USC, which can be targeted by FDA-approved repositioned compounds. We demonstrated that high levels of ryanodine receptor 1 (RYR1), a Ca^{2+} release channel is associated with poor OS in patients with USC. We further revealed the molecular mechanism by which RYR1 modulates the malignant phenotypes of USC, and demonstrated the feasibility of using dantrolene as a repositioned drug for the treatment of the disease.

Materials And Methods

For more details, see Supplemental Experimental Procedures. Reagents and Materials are summarized in Table S3-5.

Animals and *in vivo* procedures

All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center, which approved the procedures described below. The study is compliant with all relevant ethical regulations regarding animal research. Female nude mice (NCRNU, 6 weeks) were purchased from Taconic Biosciences. For drug

treatment, luciferase-labeled ARK1 cells were suspended in 100 μ L of PBS and intraperitoneally injected into 6-week-old female nude mice. Four weeks later, mice were treated with dantrolene 5 mg/kg or vehicle every other day (N = 10/group). After 5 weeks of treatment, *in vivo* bioluminescence was detected by the IVIS-200 bioluminescence and fluorescence imaging system (Caliper Life Sciences). At week 6, mice were euthanized, and necropsy was performed. The tumor weight from each mouse was recorded. Tumor tissues were fixed in formalin and processed for histological evaluation. For knockdown experiments, ARK1 cells stably expressing RYR1 or control shRNAs were injected into 6-week-old female mice intraperitoneally. *In vivo* bioluminescence was monitored every 2 weeks, and signals were recorded. At week 10, all the mice were euthanized.

Human samples

Uterine tissue samples from the primary uterine sites of pre-treatment cases were obtained from the uterine cancer repository of the Department of Gynecologic Oncology and Reproductive Medicine at The University of Texas MD Anderson Cancer Center. Approval from the MD Anderson Institutional Review Board was obtained prior to the study.

Cell culture

Human USC cell lines ARK1, ARK2, and HEC50 were obtained from ATCC and cultured in RPMI-1640 medium (Life Technologies) with 10% fetal bovine serum (FBS) (Life Technologies) and 5 U/mL penicillin and streptomycin (P/S) (Life Technologies) at 37°C with a humidified 5% CO₂ environment. All cell lines were tested for mycoplasma contamination by MycoAlert Mycoplasma Detection Kit (Lonza) and authenticated using the microsatellites panel. Stable RYR1-knockdown and overexpressed ARK1 cells were maintained in RPMI-1640 supplemented with puromycin (1 mg/mL, Invitrogen). All cells were routinely cultured with sub-culturing every 2-3 days.

siRNAs, shRNAs, CRISPR/Cas9, and virus production

Commercially available siRNAs for human RYR1, PGC-1 α , and corresponding control siRNAs were purchased from Thermo Fisher. The efficiencies of RYR1 and PGC-1 α silencing were validated by QPCR and Western blotting. Lentiviral transduction particles of 2 shRNA oligos specially targeting 2 distinct regions within human RYR1 coding sequence were purchased from Sigma (shRYR1-1 TRCN0000174210; shRYR1-2 TRC0000359103). The knockdown efficiency and specificity of the 2 shRNAs were confirmed by QPCR and Western blotting. High-titer CRISPR/dCas9 human RYR1 lentiviral activation particles and control particles were purchased from Santa Cruz (Santa Cruz, #sc-422774-LAC; #sc-437282). RYR1 CRISPR/dCas9 activation particles work through a synergistic activation mediator transcription activation system containing a deactivated Cas9 (dCas9) nuclease, activation complex, and target-specific guide RNA. Following transfection, RYR1 expression was examined by QPCR and Western blotting.

Cell transfection and lentiviral-based gene transduction

For siRNA experiments, cells were plated 1 day before siRNA (~50 μ M) transfection with Lipofectamine RNAiMAX reagent in Opti-MEM reduced serum medium (Invitrogen) for 24-48 h. For the generation of stable RYR1 knockdown cell lines, lentiviral particles of shControl, shRYR1-1 and shRYR1-2 were used to infect ARK1, ARK2, and HEC50 at the multiplicity of infection 5 for 24 h. Transduced cells were screened by RPMI-1640 containing 3 μ g/mL of puromycin (GIBCO) for approximately 7 days until all control cells died. Stable cells derived from single-cell colonies were expanded, frozen, and subjected to indicated experiments. For the generation of stable RYR1-overexpressing cell lines, ARK1, ARK2, and HEC50 were seeded in a 96-well cell culture plate, 4000 cells/well, 24 h prior to virus transduction. Human RYR1 lentiviral particles or control particles were incubated with ARK1, ARK2, and HEC50 cells at the multiplicity of infection 5 for 24 h, followed by puromycin (3 μ g/mL) selection for positive clones according to the manufacturer's protocol.

Cell viability, migration, and apoptosis

Equal numbers of cells were seeded in 96-well plates in triplicate at a density of 4000-5000 cells per well. Cell viability was measured by incubation with MTT reagent (Sigma) at a final concentration of 0.5 mg/mL for 1 h. Absorbance was measured at the wavelength of 570 nm by a microplate reader (BMG LABTECH). Optionally, cell viability was also analyzed using real-time cell analysis (RTCA) with the xCELLigence system E-Plate (ACEA Biosciences). The cell suspension was then added to the medium and incubated after the background cell index (CI) values were recorded. Dantrolene sodium (Abcam) was added when the cell was attached. Cell proliferation was observed at 10-min intervals using the RTCA analyzer. For cell migration, transwell assay was performed: 2000 cells were suspended in FBS and P/S free RPMI-1640 and seeded in the upper chambers of a 24-well transwell device (ThermoFisher). Complete RPMI-1640 (10% FBS) was filled in the lower chambers. After 24-72 h, migrated cells were stained and counted. Cells were planted in a 6-well plate until confluence, and at least 3 scratches were made for each sample by a pipette tip. Cell migrated distance was measured after 0, 12, 24, 48, and 72 h. Apoptotic cells were detected by annexin V and propidium double staining, followed by flow cytometry using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) according to manufacturer's protocol.

Microdissection of tissue samples and transcriptome profiling

Tumor and normal epithelial cells were procured by microdissection as previously described [10]. In brief, tissue sections were fixed in 70% ethanol and stained with 1% methyl green. Areas with infiltration by immune cells or blood vessels were excluded to minimize contamination during microdissection. Total RNA was extracted using the PureLink RNA Min Kit (Ambion). GeneChip Human Genome U133 Plus 2.0 microarray (Affymetrix) was applied to hybridize purified and labeled RNA samples according to the manufacturer's protocol. An Affymetrix Fluidics Station 450 and GeneChio Scanner 3000 7G (Affmetrix) were employed for staining arrays.

Droplet digital polymerase chain reaction (ddPCR)

Purified RNA was used to synthesize the cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). ddPCR analysis was performed using ddPCR Supermix for Probes (no dUTP) (Bio-Rad) on a C1000 Touch Thermal Cycler (Bio-Rad). Droplet was generated by Automated Droplet Generator for Droplet Digital (Bio-Rad). The result was read using the QX200 Droplet Reader (Bio-Rad). dd-PCR was performed using the following probe: RYR1: s00166991_m1; RYR2: Hs00181461_m1; RYR3:Hs00168821_m1 (ThermoFisher).

Reverse-phase protein array process (RPPA)

RPPA analysis was performed as described previously with some modifications [11]. In brief, cell pellets were collected and washed. Total proteins were extracted by RPPA lysis buffer. Protein lysis was printed on nitrocellulose-coated slides by using Aushon Biosystems 2470 arrayer. 300 ~ 500 validated primary antibodies were probed and examined by biotinylated secondary antibodies. Signals were amplified by a Cytomation-catalyzed system of avidin-biotinylated peroxidase (Vectastain Elite ABC kit, Vector Lab). Afterwards, signals were visualized by a streptavidin-conjugated HRP and DAB colorimetric reaction and analyzed by Array-Pro Analyzer software (MediaCybernetics).

Western blotting analysis

Cells or tumor tissues were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors. Protein concentration was measured by BCA assay (Life Technologies). Equal amounts of protein were denatured, subjected to SDS-PAGE and immunoblot assay using indicated primary antibodies overnight at 4°C. Specific antibody binding was detected by peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Signals were visualized by chemiluminescence detection kit (Bio-Rad). Beta-actin was applied as a loading control. Density analysis of immune-bands was performed by ImageJ software.

ATP measurement

CellTiter-Glo 2.0 assay (Promega) was performed to examine ATP production in live cells. According to the manufacturer's protocol, the standard curve showing the correlation between cell number and luminescent output was first generated. Serial dilutions of cells with 0, 2500, 5000, 10000, 15000, and 20000 cells per well were planted in a 96-well plate. Equal volume of CellTiter-Glo 2.0 reagent was added to each well. After 10 minutes, luminescence was recorded by a microplate reader (BMG LABTECH). For RYR1 knockdown and overexpression stable cell lines, 8000 cells per well were seeded in an opaque-walled 96-well plate. After 24 h, 50 µL CellTiter-Glo reagent was added in each well, which contained an equal volume of culture medium, followed by 10 min incubation and signal recording. For drug treatment, cells were seeded in a 96-well plate 24 h prior to treatment with dantrolene at concentrations of 50 µM or DMSO, and ATP measurement was taken at 0, 24, 48, and 72 h. Stable RYR1-overexpressing cells, cultured in complete RPMI-1640, were incubated with CBP-AKT inhibitor (Tocis #5773), CREB interaction inhibitor (Sigma #217505), or DMSO as control. ATP was checked after treatment of 24, 48, and 72 h by CellTiter-Glo 2.0 assay. PGC-1α was silenced in RYR1 knock-in cells using PGC-2a specific siRNAs. ATP

level in PGC-1a silenced cells were estimated after transfection for 48 h using CellTiter-Glo 2.0 assay. To measure ATP level in tumor tissues, ATP Colorimetric/Fluorometric Assay Kit was applied (BioVision #K354). In brief, 20 mg homogenized tissues were lysed in 100 μ L ATP Assay Buffer, followed by deproteinization using a deproteinizing sample preparation kit (BioVision #K808) based on the manufacturer's instructions. Reaction mix (50 μ L) was incubated with 50 μ L of deproteinized samples for 30 min at room temperature, protected from light. Absorbance (570 nm) or fluorescence (535/587 nm) of standards and samples were measured. ATP amount in tumor tissues were calculated after the sample readings were applied to ATP standard curve according to manufacturer's protocol.

Intracellular and mitochondrial ROS measurement

Cell lines were treated with 50 μ M dantrolene or DMSO for 72 h. Stable cells were planted 24 h prior to the analysis. Cells were then digested, washed, and stained by H2DCFDA (BioVision) for 30 min at 37°C protected from light. Intracellular ROS was estimated via mean fluorescence intensity in Ex/Em 495/529 nm using flow cytometry and compared among different sample groups according to the manufacturer's description. Mitochondrial ROS was measured using Mitochondrial ROS Detection Assay Kit (Cayman). Cells were stained for 1 h at 37°C in the dark, and analyzed by flow cytometry (Ex/Em: 480-515 nm/560-600 nm).

Oxygen consumption (OCR) and extracellular acidification rate (ECAR) analysis

OCR, ECAR, and linked ATP production rates were measured by Agilent Seahorse XF Real-Time ATP Rate Assay Kit (Agilent #103592-100). Briefly, appropriate numbers of cells were seeded (6 replicates) in XF96 Cell Culture Microplates (Seahorse #) at 70%-80% confluency in complete RPMI-1640 prior to the analysis. After 24 h, cell culture medium was replaced by Seahorse XF RPMI medium, pH 7.4 with 10 mM of XF glucose, 1 mM of XF pyruvate, 2 mM of XF glutamine (Agilent #103576-100), and cells were incubated in a non-CO₂ incubator at 37°C for 45-60 min. The cartridges were preincubated with calibration solution (Agilent #100840-000) at 37°C in a non-CO₂ incubator overnight. Seahorse XF96 analyzer was used for monitoring OCR and ECAR values after injections of oligomycin and rotenone/antimycin A. OCR and ECAR were normalized to cell number and protein amount and analyzed by WAVE software (Agilent).

Targeted metabolomics analysis

For cultured cells, a total of 1×10^7 cells in optimal conditions were seeded in complete RPMI-1640 medium in T75 flasks. After 24-48 h, cells were digested, washed, counted, and frozen in liquid nitrogen or immediately subjected to metabolites extraction and targeted metabolomics analysis for glycolysis and TCA metabolites. For tumor tissue samples, at least 100 mg of fresh or frozen tumor tissues from treated or control groups were digested and submitted for measurement of glycolysis and TCA metabolites. The targeted metabolomics analysis was executed using the protocol described previously [12] (Supplementary Experimental Procedures).

Mitochondrial membrane potential assay

The mitochondrial membrane potential ($\Delta\Psi_m$) was measured by the JC-1 kit (ThermoFisher#M34152) based on the manufacturer's instructions. In general, 2×10^6 cells were seeded in a 35mm culture dish and treated with dantrolene or DMSO for 72 h. Stable cells were planted 24 h prior to the analysis. Cells were collected and washed with PBS, followed by stained with JC-1 solution for 30min at 37°C. The fluorescence intensity was detected with a flow cytometry at 514/529 nm (Green) and 585/590 nm (Red).

NAD⁺/NADH assay

NAD⁺ and NADH in human whole cell lysates were measured by NAD/NADH-Glo™ kit (Promega#G9071) following the manufacture's protocol. Briefly, 6000-7000 cells per well were seeded into a 96 well plate 24-72 h before measurement. Cells were lysed in bicarbonate buffer with 1% dodecyltrimethylammonium bromide (DTAB). To quantify NAD⁺, half of the lysate was mixed with 0.4N HCl to remove NADH and heated at 60°C for 15 mins, followed by adding Trizma base buffer. The other half of cell lysate was heated at 60°C for 15 mins and mixed with HCl/Trizma buffer to remove NAD⁺. Equal volume of NAD/NADH-Glo detection reagent was added to acid or base treated cell lysates and luminescence was recorded using a luminometer.

Quantification and statistical analysis

The SPSS 24 (IBM Corp) and GraphPad Prism 8.0 (GraphPad Software) software programs were used to perform the statistical tests. All of the *in vitro* experiments were set up at least 3 samples/repeats per experiment/group/condition. Five fields per slide were selected randomly for the quantification of IHC staining. For mouse experiments, 6-10 mice per group were used for the analysis. Quantitative data were expressed as mean $\pm$ SEM of the indicated number of experiments. Statistical differences were tested by the 2-tailed Student t-test and 1-way or 2-way ANOVA. The Mann-Whitney U test was used for the analysis of nonparametric data. Benjamini-Hochberg FDR multiple testing corrections and t-test were used for microarray data analysis. A p value < 0.05 was considered statistically significant. Pearson's correlation test was used to evaluate the correlation coefficient between 2 parameters. Survival studies were performed by using Kaplan-Meier curves, and p values were calculated by the log-rank test. TCGA data were obtained from a public repository (<https://www.cbioportal.org/>). GSE USC patients' cohorts were extracted from the GEO online database (<https://www.ncbi.nlm.nih.gov/geo/>).

Results

RYR1 is an unfavorable prognostic marker for USC

To exploit the promising therapeutic target candidates with prognostic significance in USC, we first identified prognostic markers specific for USC using Significance Analysis of Microarray (SAM) on The Cancer Genome Atlas (TCGA) endometrial cancer datasets. A total of 2064 and 1014 differentially

expressed genes were identified between normal controls vs USC and endometrioid endometrial cancer (EEC) vs USC, respectively (Figure 1A). In addition, 1467 genes were significantly correlated with OS in USC patients by COX regression analysis. Among these genes with prognostic significance, 37 genes overlapped with those that showed significantly higher expression in USC than normal control, and EEC, suggesting that they represent prognostic markers specific for USC. Among them, a DrugBank search identified only RYR1, which can be targeted by FDA-approved drugs (Table S1). Figure 1B shows the overall survival of 56 advanced stage USC patients in TCGA dataset with 22 high RYR1 and 34 low RYR1 (z-score = 1.08 as cutoff, $p = 0.031$).

Next, we asked whether USC cells demonstrated markedly higher RYR1 expression levels than normal counterparts *in vitro*. ddPCR showed that all USC cell lines (ARK1, ARK2, and HEC50) had significantly higher RYR1 mRNA levels than EEC cell lines (HEC-1A, HEC-1B, HEC-59, and ECC) and normal uterine epithelial (UE) cells (Figure 1C). Western blot demonstrated markedly higher RYR1 protein in all USC than UE cells (Figure 1D).

RYR1 promotes tumor progression and inhibits apoptosis in USC

RYR1 encodes the ryanodine receptor type 1 protein predominately found in skeletal muscle cells [13]. It functions as a Ca^{2+} release channel located in the membrane of sarcoplasmic reticulum (SR, muscle cells) or endoplasmic reticulum (ER, non-muscle cells) to mediate the release of Ca^{2+} from the SR/ER. Several drugs or compounds are known to modulate RYR1 activity, such as dantrolene [14], ryanodine [15], ruthenium red [16] and others. Of these, dantrolene is the only specific RYR1 antagonist that is approved by FDA for the treatment of malignant hyperthermia [17]. To determine the role of RYR1 in conferring malignant phenotypes in USC, stable knockdown of RYR1 was performed in USC cell lines ARK1, ARK2 and HEC50 cells using RYR1-specific small hairpin RNA (shRNA), and RYR1 overexpression was generated by transfecting the full-length RYR1 cDNA construct. Knockdown and overexpression of RYR1 were confirmed by ddPCR and Western blot, respectively (Supplementary Fig. S1A, B). MTT assay demonstrated that RYR1 silencing suppressed cell proliferation in the 3 USC cell lines with higher proliferation rates in RYR1-overexpressing cells (Figure 2A, B). Depletion of RYR1 caused a reduction in USC cell migration as detected by the transwell assay (Figure 2C). The apoptotic cell ratio was increased in USC cell lines after RYR1 knockdown as assessed by flow cytometry (Figure 2D) with contrast results in RYR1 overexpressing cells (Supplementary Fig. S1C, D). These findings indicate RYR1 is a potential common pharmaco-therapeutic target in USC.

We examined whether dantrolene could potentially be repurposed for the suppression of the USC phenotypes, as observed in RYR1 shRNA-silenced USC cells. Application of dantrolene exhibited a remarkable inhibitory effect on the proliferation and mobility of USC cells (Figure 2E-F). Furthermore, flow cytometry demonstrated that dantrolene-treated USC cells had significantly higher numbers of apoptotic cells than controls (Figure 2G). To confirm the on-target effect of dantrolene, RYR1-silenced ARK1 cells were treated with dantrolene (50 μM) or vehicle solution, and the cell growth was monitored. Significant difference inhibitory effect of dantrolene was observed in the mock transfectants instead of RYR1

shRNA-transfected ARK1 cells (Supplementary Fig. S1E). These findings imply that RYR1 mediates the effect of dantrolene in suppressing the malignant phenotype in USC.

To examine the tumor-promoting effect of RYR1 *in vivo*, luciferase-labeled RYR1 shRNA or mock transfected ARK1 cells were intraperitoneally injected into 6-week-old female nude mice. Tumor progression was examined by measuring bioluminescence every 2 weeks (Figure 2H). Mice injected with RYR1-silenced ARK1 cells had significantly lower bioluminescence signals detected at week 10, compared to those injected with control cells (Figure 2I). Survival analysis showed that mice injected with RYR1-silenced ARK1 cells had significantly longer OS times than controls (Figure 2J), supporting the hypothesis that RYR1 plays a crucial role in USC progression. Similarly, mice were injected intraperitoneally with dantrolene (5 mg/kg) or vehicle 3 times per week for 5 weeks, 28 days after luciferase-labeled ARK1 cell inoculation to check dantrolene effect *in vivo*. Dantrolene-treated group had significantly lower bioluminescence signals than control group (Figure 2K). There was no significant difference in the body weight of dantrolene- and vehicle-treated mice (Supplementary Fig. S1F), suggesting minimal toxicity of dantrolene in the mice.

To further determine the effect of RYR1 silencing and dantrolene treatment on tumor cell proliferation, angiogenesis, and apoptosis, immunolocalization (IHC) of Ki-67, CD31, and cleaved caspase 3 (CCP3) were performed on tumor tissues harvested in the mouse models. USC tumors of the RYR1 knockdown group and the dantrolene-treated group had significantly lower numbers of Ki-67- and CD31-positive cells and higher numbers of CCP3 cells compared to controls (Supplementary Fig. S1G, and Figure 2M-N).

Taken together, our results strongly support the tumorigenic role of RYR1 in USC and the feasibility of repurposing dantrolene as an effective agent for the treatment of USC.

RYR1 modulates intracellular calcium signaling in USC cells

To investigate the RYR1-dependent changes in Ca^{2+} homeostasis of USC cells, cytosolic $[\text{Ca}^{2+}]$ was first determined by radiometric measurement of Fura-2 fluorescence in ARK1-shCtrl, ARK1-shRYR1, ARK1-Control and ARK1-RYR1 cells. The resting $[\text{Ca}^{2+}]_i$ was significantly suppressed in the shRYR1-treated cells (shCtrl: 99.4 ± 2.1 nM, $n = 58$; shRYR1: 74.4 ± 3 nM, $n = 87$; $p < 0.001$) and significantly elevated in RYR1-overexpressing cells (Control: 94 ± 3.2 nM, $n = 171$; RYR1: 104.5 ± 2.2 nM, $n = 217$; $p < 0.001$; Figure 3A), suggesting the differences in resting $[\text{Ca}^{2+}]_i$ in the indicated cells were related to Ca^{2+} signals mediated by RYR1.

The activity of RYRs was further examined by the RYR agonist 4-Chloro-m-cresol (4-CMC). Application of 4-CMC (5 μM) caused a rapid transient increase in cytosolic $[\text{Ca}^{2+}]$. The responses were significantly suppressed in the shRYR1 cells and accentuated in the RYR1-overexpressing cells (Figure 3B). These results suggested that functional RYR1 is expressed and operated as an important determinant of Ca^{2+} homeostasis in ARK1 cells. RYR1 may modulate mitochondrial $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_m$) through ER-mitochondrial Ca^{2+} transfer [18]. ER-mitochondrial Ca^{2+} transfer was monitored in ARK1 cells transfected with the

mitochondrial Ca^{2+} biosensor CEPIA2mt (Figure 3C). Application of 4-CMC caused a rapid increase in $[\text{Ca}^{2+}]_m$ in the control ARK1 cells. The response was enhanced in the RYR1-overexpressing cells and was significantly suppressed in ARK1 cells transfected with shRYR1 (Figure 3B-C). In a separate set of experiments, ARK1 cells were transfected with both the ER specific Ca^{2+} biosensors G-CEPIA1er and CEPIA2mt for simultaneous recording of ER $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_{ER}$) and $[\text{Ca}^{2+}]_m$, respectively. Activation of RYRs by photorelease of caged cADP-ribose (cADPR), an endogenous activator of RYRs, at a subcellular region of interest (ROI) caused an immediate transient reduction in $[\text{Ca}^{2+}]_{ER}$, which was associated with a fast, transient increase in $[\text{Ca}^{2+}]_m$ (5-10 s) followed by a reduction in the mitochondrial Ca^{2+} signal (Figure 3D-E). The ER-mitochondrial Ca^{2+} transfer occurred locally within the ROI undergoing photolysis, without affecting $[\text{Ca}^{2+}]_{ER}$ or $[\text{Ca}^{2+}]_m$ in the other ROIs. These experiments clearly demonstrated that RYR is capable to mediated ER-mitochondrial Ca^{2+} transfer in ARK1 cells.

RYR1 silencing down-regulates mitochondrial genes in USC

To identify the RYR1-mediated signaling network that modulates the malignant phenotypes of USC, unbiased reverse-phase protein array (RPPA) was performed on over 400 proteins of key signaling networks. Cell lysates from RYR1 shRNA and scramble shRNA-transfected ARK1 cells as well as dantrolene- and vehicle-treated ARK1 cells were used to identify the overlapping differentially expressed proteins that are associated with down-regulation of RYR1 expression and activity. A total of 222 and 233 differentially expressed proteins were found. Among them, 64 downregulated and 38 upregulated proteins were common in both experimental treatments (Supplementary Fig. S2A).

Our attention was drawn to proteins in the top rank of the overlapping list (Figure 4A-B). They included components of mitochondrial electron transport chain (mETC) (e.g., COXIV, NDUFB4, ATP5a, SDHA), suggesting the potential role of RYR1 in modulating mitochondrial functions. The RPPA results were validated by qPCR and Western blot demonstrating that the USC cells stably transfected with RYR1 shRNA had significantly lower mRNA and protein levels of NDUFB4 (Complex I), SDHA (Complex II), COX-IV (Complex IV) and ATP5a (Complex V) than the mock transfectants (Figure 4C-F). Furthermore, dantrolene (50 μM) treatment of USC cells caused significant time-dependent reduction in these mETC gene mRNA levels (Figure 4G-I).

To further determine the role of RYR1 in regulating mETC gene expression in USC tissue samples, regression analysis was performed using TCGA datasets to determine the correlation between mRNA expression of RYR1 and the mETC genes. The results showed significantly higher expression of these 3 mETC genes in USC than in normal control (Figure 4J); and significant positive correlations were observed between the expression of RYR1 and the mETC genes in USC, except COXIV (Figure 4K). In addition to the above 4 mETC genes, we found positive correlations between RYR1 and some other members of NDUFB, SDHA, COX, and mitochondrial ATPase subfamilies in GEO datasets (GSE24537) (Supplementary Tables S2).

Furthermore, mRNA expression levels of NDUFB4, SDHA, and ATP5a, as well as some other members were significantly higher in USC than normal controls in patient cohorts from GEO (Supplementary Fig. S2B-C), suggesting that these mETC genes play a crucial role in mediating the tumor-promoting effect of RYR1 in USC.

RYR1 modulates mitochondrial bioenergetics properties in USC

Our results demonstrated that RYR1 regulated the expression of key mETC components (Figure 5A) in USC and HGSOC cells, suggesting that RYR1 may modulate mitochondrial bioenergetics in these cancer cells. To test this hypothesis, the RYR1-dependent regulation of oxidative phosphorylation (OXPHOS) was determined in ARK1 cells. Oxygen consumption rate (OCR) and ATP production rate were first determined by a Seahorse Analyzer. The results showed that there was significant reduction in OCR, the basal mitochondrial and whole cell ATP production rates (pmol/min) in shRYR1-transfected or 50 μ M dantrolene-treated ARK1 cells (Figure 5B-E). In contrast, ATP production rate was significantly higher in RYR1 stably transfected cells than the mock transfectants (Figure 5F).

Next, we determined total live-cell ATP production and mitochondrial membrane potential (MMP) in USC cells using the CellTiter-Glo kit and the JC-1 assay. Tetrachloro-teraethyl benzimidazole carbocyanine iodide (JC-1) is a cationic dye that accumulates in mitochondria, which is commonly used for monitoring MMP ($\Delta\psi_m$). Usually, under low MMP, JC-1 exists as monomer and yields green fluorescence. The dye will aggregates under MMP leading to red fluorescence [19]. RYR1 depleted ARK1 cells had significantly lower total live-cell ATP and MMP (Figure 5G, H) with contrast result in RYR1 overexpression cells (Figure 5I and Supplementary Fig. S3A). Furthermore, USC cell lines treated with dantrolene (50 μ M) for different durations showed that dantrolene decreased live-cell ATP levels in a time-dependent manner (Figure 5J). A significant decrease in MMP was also observed in dantrolene-treated USC cells (50 μ M) (Figure 5K) and ARK1-shRYR1 cells induced mouse tumor tissues (Figure 5L) also demonstrated a reduced ATP production compared to control.

To further examine the role of RYR1 on OXPHOS, the effect of RYR1 expression on 2 additional key OXPHOS-related parameters—NAD⁺/NADH ratio and accumulation of reactive oxygen species (ROS) were determined. The results showed significantly higher NAD⁺/NADH ratio, mitochondrial and total cellular ROS in RYR1-silenced or dantrolene-treated USC cells than control cells (Figure 5M-P) with contrast results in RYR1-overexpressing cells (Supplementary Fig. S3B-C). Taken together, these results demonstrate that RYR1 alters mitochondrial biogenetics properties through modulating OXPHOS process in USC.

RYR1 silencing suppresses glycolysis and TCA cycle in USC

To exploit energy metabolism alterations driven by RYR1, targeted metabolomics analysis was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in RYR1-deficient ARK1 cells and control cells. The results revealed a markedly lower concentration of glycolytic and TCA metabolites, such as G6P/F6P, FBP/GBP, citrate, succinate and malate in both RYR1 knockdown and 50 μ M dantrolene-

treated cells, suggesting that removal of RYR1 impeded the normal operation of the glycolysis and TCA cycle in ARK1 cells (Figure 6A-C). Extracellular acidification rate (ECAR), which measures the excretion of lactic acid per unit time in RYR1 knockdown and dantrolene-treated ARK1 cells was determined. ECAR of the shRYR1-treated ARK1 cells was significantly lower than controls (Figure 6D).

Interestingly, we found that the glutamine level was markedly increased in RYR1-knockdown and dantrolene-treated ARK1 cells (Figure 6A-B), suggesting that increased glutamine uptake could be a feedback mechanism by which the cells compensated for the shutting down of both OXPHOS and glycolysis due to RYR1 depletion. In congruent with the LC-MS experiments, glutamine level was significantly elevated after RYR1 silencing or blockage (Figure 6E-F) and decreased in RYR1-overexpressing cells (Figure 6G), suggesting that high levels of RYR1 can probably speed up glycolysis and TCA cycle, which does not need glutamine compensation.

Ca²⁺ dependent AKT/CREB/PGC-1 α and AKT/HK1/2 axes is essential for RYR1-mediated USC progression

Peroxisome proliferator-activated receptor coactivator protein-1alpha (PGC-1 α) has been shown to bind directly to the promoter regions of mETC genes to upregulate transcription in breast and ovarian cancer cells [20]. We examined whether PGC-1 α mediated the effect of RYR1 in regulating mETC expression. Both PGC-1 α mRNA and protein levels were significantly lower in ARK1 and ARK2 cells transfected with RYR1 shRNA or treated with dantrolene (50 μ M) (Figure 7A-C). Furthermore, PGC-1 α silencing and rescue experiments were performed by overexpressing RYR1 in PGC-1 α -silenced ARK1 cells, and mETC genes expression was determined using qPCR. We found that the decrease of mETC genes in PGC-1 α -knockdown cells can be compensated by overexpressing RYR1 in those cells (Figure 7D). These results clearly suggest that RYR1 may indeed regulate PGC-1 α expression to modulate the expression of mETC genes in USC cells.

Next, we determined whether hexokinases (HKs), which are key enzymes that catalyze the first step of glucose metabolism by converting glucose to glucose-6-phosphate (G6P), mediate the effect of RYR1 in regulating glycolysis and TCA cycle. Of the HKs, HK1 and HK2 are the 2 predominant isoforms in most human tissues [21]. Western blot showed markedly lower levels of total HK1 and HK2 in RYR1-silenced ARK1 cells than in control cells (Figure 7E). To further determine the molecular mechanism by which PGC-1 α and HK1/2 are regulated by RYR1, we screened all the known Ca²⁺-dependent signaling molecules and transcription factors from our RPPA and public datasets. We focused on Ca²⁺/cAMP response element (CRE)-binding protein (CREB), which upon activation can form a functionally active dimer that binds to the cis-acting CRE element within the promoters of target genes. The PPARGC1A gene contains a CREB binding domain within its promoter. CREB phosphorylated by AKT is able to directly interact with this domain and trigger PGC-1 α transcription [22]. Our RPPA data demonstrated that RYR1 shRNA-transfected and dantrolene-treated ARK1 cells had significantly lower levels of phosphorylated AKT and CREB than controls (Supplementary Fig. S4A). This finding was validated by Western blot (Figure 7F).

To further confirm that AKT and CREB play a role in regulating PGC-1 α expression, Western blot were performed on ARK1 cells treated with either AKT inhibitor Akti-1/2 or CBP/CREB inhibitor. The results showed markedly lower phosphorylated AKT (pS473) and CREB (pS133) levels as well as PGC-1 α levels after treatment of Akti-1/2 or CBP/CREB inhibitor (Supplementary Fig. S4B). Western blot showed that both HK1 and HK2 expression were reduced in a dose-dependent manner after treatment with Akti-1/2 but not CBP/CREB inhibitor (Supplementary Fig. S4C), suggesting that HK1/2 were specifically regulated by the CREB-independent AKT pathway. Furthermore, RYR1-overexpressing ARK1 cells were treated with Akti-1/2 or CBP/CREB inhibitor and controls followed by Western blot for PGC-1 α . The results showed that the effect of RYR1-enhanced PGC-1 α expression was abrogated by Akti-1/2 and CBP/CREB inhibitor (Figure 7G), suggesting that RYR1 modulates PGC-1 α expression through the AKT/CREB/PGC-1 α pathway. To confirm that PGC-1 α mediates the promoting effect of RYR1 on ATP production and mETC gene expression, PGC-1 α -specific siRNAs or control siRNA was transfected into USC cells with or without stably overexpressing RYR1. The silencing efficiency of PGC-1 α siRNAs in USC cells was validated by qPCR (Supplementary Fig. S4D). Cells transfected with full-length RYR1 and control siRNA had significantly higher levels of ATP production, which was reversed by treatment with PGC-1 α siRNAs (Figure 7H). In addition, the levels of mETC genes were also significantly higher in the RYR1-overexpressing cells compared to the mock transfectants. The enhanced expression of mETC genes in RYR1-overexpressing cells was abrogated by Akti-1/2, CBP/CREB inhibitor, and PGC-1 α siRNA in ARK1 cells (Figure 7I). These results established the causal relationship of the RYR1-dependent AKT/CREB/PGC-1 α pathway with elevated ATP level and mETC gene upregulation. In addition, the enhanced cell proliferation rate induced by RYR1 overexpression in ARK1 cells was abolished by inhibitor treatment (Fig. 7J).

To further confirm that RYR1-dependent regulation of the AKT/CREB/PGC-1 α pathway is dependent on Ca²⁺ signals modulated by RYR1, [Ca²⁺]_i was buffered by incubating ARK1 cells with the cell permeant Ca²⁺ chelator BAPTA-AM (10 μ M) for 1, 12, and 24 hours. The levels of p-CREB (S133) and p-AKT (S473) were significantly lowered in cells incubated with BAPTA-AM, accompanied with a significant reduction in the level of PGC-1 α . The decrease in p-CREB (S133), p-AKT (S473), and PGC-1 α protein levels after BAPTA treatment was attenuated in RYR1 knockdown ARK1 cells (Figure 7K and Supplementary Fig. S4E). Additionally, the enhanced phosphorylation of AKT and CREB and overexpression of PGC-1 α were again effectively obliterated by the Ca²⁺ chelators (Supplementary Fig. S4F). The mRNA level of PGC-1 α induced by RYR1 overexpression was also attenuated by BAPTA treatment (Supplementary Fig. S4G). These results provide further evidence that RYR1-dependent Ca²⁺ signals mediate the enhanced AKT and CREB activities, leading to the upregulation of PGC-1 α .

Discussion

In this study, we sought to identify unfavorable prognostic biomarkers of USC that can be targeted by FDA-approved compounds and to investigate their underlying mechanisms for tumor growth. The major findings are, first, a meta-analysis of patient databases found that RYR1 is upregulated in USC tumors

and its upregulation is associated with abbreviated survival of USC patients. Second, RYR1 is ubiquitously upregulated in USC cell lines; inhibition of RYR1 suppressed proliferation and enhanced apoptosis. Third, RYR1 upregulation is associated with increased resting $[Ca^{2+}]_i$, enhanced SR Ca^{2+} release and effective ER-mitochondrial Ca^{2+} transfer. Fourth, depletion or inhibition of RYR1 in USC cell lines revealed important RYR1-dependent gene regulation of mitochondrial ETC and glycolysis and casually related to alterations in bioenergetics properties. Fifth, the aberrant mitochondrial and glycolytic activities in the USC cells are mediated by RYR1 via the Ca^{2+} -dependent AKT/CREB/PGC-1 α and AKT/HK1/2 pathways. These results for the first time revealed the important roles of RYR1 in USC tumor progression, elucidated the RYR1-dependent pathological mechanisms, and suggested RYR1 as a potential target for the treatment of USC. Hence, repurposing the FDA-approved RYR1 blocker dantrolene can be an attractive additional therapy for USC.

RYR dysfunction has been linked to several detrimental diseases [23], including malignant hyperthermia [24] and catecholaminergic polymorphic ventricular tachycardia [25]. Functional RYRs has been reported in T-lymphoma cells [26], prostate cancer cells [27], neuroblastoma [28], and breast cancer cells [29, 30]. However, there is no related report on the role of RYRs, particularly RYR1, in the pathogenesis of USC. In this study, we found that RYR1 is the only RYR subtype upregulated in USC and their cell lines. Its upregulation is associated with altered Ca^{2+} homeostasis. The elevated $[Ca^{2+}]_i$ is apparently related to the increased Ca^{2+} release from RYR1, and probably RYR-dependent Ca^{2+} influx via the store-operated Ca^{2+} entry mechanism [31]. Increasing evidence suggests important roles of Ca^{2+} signaling in cancer pathogenesis [32]. RYR1 may represent a major aberrant Ca^{2+} mechanism in USC because its expression and activity are strongly linked to the malignant phenotypes of enhanced proliferation, migration, and apoptosis resistance.

RYR1 can exert its mitogenic influence through multiple signaling mechanisms. It provides diverse Ca^{2+} signals for the activation of Ca^{2+} -sensitive pathways to trigger tumorigenic gene expression. Our data showed that the expression level of mETC enzymes were strongly dependent on the availability of RYR1. The reduction of OXPHOS, oxygen consumption, ATP production, mitochondrial membrane depolarization and increase in ROS level in RYR1-depleted or dantrolene-treated USC cells were also observed. Altered mitochondrial bioenergetics and biosynthetic states have long been implicated in tumorigenesis [33]. In USC, there are frequent mutations in somatic D-loop mitochondrial DNA, which contains essential transcription and replication elements; mutations in this region might alter the mitochondrial functions [34]. Hence, our results provide the first evidence that RYR1 is responsible, at least in part, for the regulation of mitochondrial mETC activity and metabolism in USC.

Analysis of signaling pathways showed that RYR1 regulates mETC enzyme expression through the AKT/CREB/PGC-1 α pathway. PI3K/AKT activation is well recognized as an important signaling pathway, regulating cell growth, motility, survival, and metabolism in USC cells [35, 36]. Studies showed that PI3K/AKT is persistently activated in USC tissues [37], and its hyperactivation is thought to be a significant contributor to chemotherapy resistance in ovarian cancer [38]. RYR1-dependent increase in

[Ca²⁺]_i can activate the PI3K/AKT pathway by Ca²⁺/calmodulin binding to the SH3 domain of PI3K p85 subunit [39], leading to the conversion of PIP2 to PIP3, which binds to both PDK1 and AKT to facilitate phosphorylation of T308 in the “activation loop”, and subsequent phosphorylation at S473 to stimulate full AKT activity. The activated AKT phosphorylates CREB at S133 [40] and stimulates PGC-1 α expression via a CREB-dependent mechanism. Moreover, CREB can be activated synergistically by the increased [Ca²⁺]_i through CaMKIV [41]. This RYR1-dependent pathway was effectively disrupted by the suppression of RYR1 expression and activity; chelation of intracellular Ca²⁺ with the fast Ca²⁺ buffer BAPTA; inhibition of AKT or CREB with Akti-1/2 or CBP/CREB inhibitor, respectively. Thus, our results provide direct evidence for each major step in the RYR1-mediated activation of the AKT/CREB/ PGC-1 α signaling cascade and its downstream regulation of mETC expression.

In addition to the transcriptional regulation of mETC enzymes, RYR1-dependent Ca²⁺ signals can modulate mitochondrial metabolism through “ER-mitochondrial Ca²⁺ transfer” [42]. Ca²⁺ released from SR/ER enters mitochondria through voltage-dependent anion channels in the outer membrane and mitochondrial Ca²⁺ uniporters in the inner mitochondrial membrane [43, 44]. The Ca²⁺-dependent stimulation of OXPHOS provide the H⁺ gradient to maintain the mitochondrial membrane potential for ATP synthesis by F₁/F₀ ATPase, and inhibition of RYR1 in the cancer cells led to mitochondrial membrane depolarization and reduction in ATP production [45]. Moreover, suppression of RYR1 activity led to dramatic increase in ROS production, presumably through induction of mitochondrial permeability transition pore opening [46], and apoptosis. Several studies have demonstrated that elevated [Ca²⁺]_m plays important roles in cancer progression by promoting proliferation, cell migration and conferring apoptosis resistance [47–49]. This study demonstrated the RYR-mediated ER-mitochondrial Ca²⁺ transfer in USC cells by simultaneous recording of ER and mitochondrial Ca²⁺; and established its acute influences on OXPHOS, ATP production, and ROS production by inhibiting RYR1.

It is noteworthy that RYR1-dependent AKT activation contributes to enhanced aerobic glycolysis. This is evidenced by the fact that RYR1 shRNA and dantrolene reduced glycolytic intermediate metabolites and lactic acid production. Moreover, RYR1 knockdown and inhibition of AKT with Akti-1/2 significantly reduced the expression of the hexokinases HK1 and HK2. This is consistent with the well-documented role of AKT in the regulation of mitochondrial hexokinases [50] and the reports that enhanced HK2 activity is an important mechanism for tumor survival [51].

The abovementioned findings in USC cell lines are corroborated by our observations of USC in xenograft mouse models *in vivo*. RYR1 suppression in the USC xenograft mouse models dramatically reduced tumor mass. RYR1 suppression can be applied therapeutically for the treatment USC, which is supported by the test-of-concept experiment showing that the RYR1 antagonist dantrolene could effectively reduce USC progression in the xenograft mouse models.

Our study demonstrates the first time the feasibility of repurposing dantrolene in cancer treatment. In this study, dantrolene was administered by intraperitoneal injection of 5 mg/kg every 2 days, which is

equivalent to 0.4 mg/kg for an average person of 70 kg, taking into account the surface/mass ratio [52]. Hence, the effective dose of dantrolene for USC treatment could be considerably lower than that used for acute malignant hyperthermia. The low effective dose of dantrolene has the benefit of reducing the possible side effects of muscle weakness, dizziness, diarrhea, nausea, and diarrhea that may occur in patients [53]. In fact, we observed no abnormalities in other organs, especially skeletal muscle and heart, in our dantrolene-treated mice.

There are several limitations remain to be solved. First, the mechanism by which RYR1 is upregulated in USC is unknown. Previous studies have identified a number of transcriptional factors for RYR1 expression in skeletal muscles [54]. Whether these factors play a role in regulating RYR1 expression in non-excitable USC cells remains to be determined. Second, chemotherapy resistance is considered a major contributor to mortality in USC patients [55]. The possible roles of RYR1 in chemoresistance, and the potential synergistic effects of dantrolene and common first-line therapeutic agents for the treatment of USC require future investigations.

Conclusions

This study has discovered the important tumorigenic roles of RYR1 in USC progression, delineated the molecular mechanism by which RYR1-mediated Ca^{2+} signaling and mitochondrial reprogramming modulate the malignant phenotype of USC, and evaluated the potential of repurposing dantrolene in the treatment of USC. Further studies on determining the efficacy of using dantrolene alone or in combination with common first-line therapeutic agents will allow the development of new therapeutic strategies in USC treatment and the improvement of patient survival rates.

Abbreviations

ATCC: American type culture collection

ATP: Adenosine triphosphate

CRE: cAMP response element (CRE)-binding protein

CREB: cAMP response element-binding protein

NAD: Nicotinamide adenine dinucleotide

ECAR: Extracellular acidification rate

EEC: Endometrioid endometrial cancer

ER: Endoplasmic reticulum

FBS: Fetal bovine serum

FDA: Food and Drug Administration

G6P: Glucose-6-phosphate

GEO: Gene expression omnibus

HGSOC: High-grade serous ovarian cancer

mETC: Mitochondrial electron transport chain

RIPA: Radioimmunoprecipitation assay

ROS: Reactive oxygen species

RPPA: Reverse phase protein array

OCR: Oxygen consumption rate

OS: Overall survival

OXPHOS: Oxidative phosphorylation

RTCA: Real-time cell analysis

RYR1: Ryanodine receptor 1

SAM: Significance Analysis of Microarray

TCA: Tricarboxylic acid cycle

TCGA: The Cancer Genome Atlas

Declarations

Ethic approval and consent to participate

Uterine tissue samples from the primary uterine sites of pre-treatment cases were obtained from the uterine cancer repository of the Department of Gynecologic Oncology and Reproductive Medicine at The University of Texas MD Anderson Cancer Center. Approval from the MD Anderson Institutional Review Board was obtained prior to the study. All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. The study is compliant with all relevant ethical regulations regarding animal research.

Consent for publication

Not applicable

Availability of data and material

The dataset used or analyzed during the current study are available from the corresponding authors on reasonable request.

Competing interests

The authors declare no competing interests.

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Author contributions

The research was conceived and designed by L.Z. and S.C.M. Most experiments were performed by L.Z. C.X.H and T.L.Y performed USC cancer cell growth (RTCA) experiments and microdissected RNA extraction, ddPCR of RYR1 mRNA level in USC cell lines, overall survival of USC and dantrolene treated USC nude mice model. K.P.Y performed all of calcium measurement experiments. S.Y.K performed TCGA dataset screen using bioinformatics and biostatistical analysis. B.C.L, V.T and K.H.L provided USC patients clinical information and FFPE samples. C.L.A-Y, S.F.B, K.H.L, K.P.Y, and J.S.K.S provided experiments suggestions for the manuscript. The manuscript was written by L. Z, S.C.M, K.P.Y and J.S.K.S.

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Figures

Figure 1

RYR1 is significantly upregulated in USC and correlates with patient survival. A, Experimental scheme for the identification of differential genes from TCGA dataset that are associated with USC development including normal controls, EECs and USCs. B, Kaplan-Meier overall survival curve of 56 USC (B, $p = 0.031$) patients with low or high RYR1 mRNA expression (log-rank test). C, Relative RYR1 mRNA levels in USC, EEC, and UE cells detected by ddPCR. D, Western blot of RYR1 protein in the indicated USC and UE cells.

Figure 2

RYR1 enhances cell proliferation and metastasis and suppresses cell apoptosis. A, B, Growth curves showing the effect of RYR1 silencing (A) using stably expressed shRNAs (shRYR1-1 and shRYR1-2) and the control shRNA (shCtrl) and of RYR1 overexpression using stably expressed RYR1 construct and empty vector control (B) in uterine serous cancer (USC) ARK1, ARK2, and HEC50 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (2-way ANOVA). C, F, Relative number of USC shRYR1 or shCtrl cells (C),

cells treated with dantrolene (20 or 50 μ M) or vehicle (F) migrating through the transwell membrane. D, G, Relative frequencies of apoptotic cells in USC shRYP1 or shCtrl cells (D), cells treated with dantrolene or vehicle (G). E, Growth curves generated by MTT analysis showing the effect of dantrolene (30 μ M) on ARK1 and ARK2 cell proliferation. H-I, Representative bioluminescence images (H) and bar charts showing relative bioluminescence intensities (I) in nude mice at week 8 after injection with luciferase-labelled ARK1-shRYP1 or -shCtrl cells. J, Kaplan-Meier analysis for overall survival in mice injected with ARK1-shRYP1 or -shCtrl. Log rank test. $p = 0.0009$. K-L, Representative bioluminescence images (K) and bar charts showing relative bioluminescence intensities (L) at week 8 in luciferase-labelled ARK1-bearing mice injected intraperitoneally with dantrolene. $N = 10$ mice per group. M-N. Representative immunohistochemical staining images (M) and bar charts (N) of numbers of Ki-67-, CD31-, CCP3-positive cells in tumor tissues from mice treated with 50 μ M dantrolene or vehicle. Scale bar, 100 μ m. In C, D, F, G, I, L, N, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, n.s., no significance (Student t-test). In A-G, N, graphs represent mean $\pm$ SEM of 3 independent experiments.

Figure 3

RYR1 modulates intracellular calcium levels. A, Cytosolic Ca^{2+} concentration (nM) monitored by Fura-2 in ARK1-shRYR1 (n = 87) or -shCtrl (n = 58) (left) and -RYR1 (n = 271) or -Control (n = 171) (right) cells. ****p < 0.0001 (Student t-test). B, Mean normalized RYR1-mediated Ca^{2+} transient activated by 4-CMC (25 μ M) in ARK1 (blue, n = 116), ARK1-shRYR1 (red, n = 70), and ARK1-RYR1 (green, n = 55) cells. C, Mitochondrial Ca^{2+} detected by CEPIA2mt in ARK1 (blue, n = 21), ARK1-RYR1 (red, n = 16), and ARK1-

shRYR1 (green, n = 13) cells activated by 4-CMC (25 μ M). D, Representative image of 2 ARK1 cells, indicating 4 different ROIs at which photorelease of cADPR was activated, and ER and mitochondrial Ca^{2+} transient was recorded. The time course of change in ER (blue) and mitochondrial (red) Ca^{2+} signals (F/F₀) recorded in the 4 ROIs depicted in the image. E, Mean normalized ER [Ca^{2+}] and mitochondrial [Ca^{2+}] activated by photorelease of caged cADPR at subcellular ROIs in ARK1 cells (n = 12 ROIs in 9 cells).

Figure 4

RYR1 depletion suppresses mETC gene expression. A, Heatmaps showing relative expression levels of mitochondrial proteins measured by RPPA in ARK1-shCtrl, - shRYR1 cells (left); or cells treated with vehicle or dantrolene (right). Signal intensities were normalized to the control groups. B, Graphic illustration of components in 4 complexes of the mitochondrial respiration chain. C-E, G-I, Relative mRNA expression of mETC genes determined by qPCR in USC cells transfected with RYR1-specific shRNAs or shCtrl (C-E), treated with 50 μ M dantrolene or vehicle (G-I). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s., no significance (Student t-test). F, Western blots showing expression levels of mETC proteins in cell lysates from ARK1-shRYR1 or – shCtrl cells. Bands were quantified by ImageJ. β -actin was used as a loading control. Data are shown as the mean $\pm$ SEM of 3 independent experiments. J, Relative expression of NDUFB4, SDHA, and ATP5a in TCGA dataset (Student t-test). K, Correlation of NDUFB4, SDHA and ATP5a with RYR1 in GSE24537. In C-E and G-I, data represent the mean $\pm$ SEM of 3 independent. HPRT was used a loading control.

Figure 5

RYR1 modulates mitochondrial bioenergetics properties. A, Graphic illustration of ETC functions in mitochondrial membrane. B-C, Representative pattern of OCR as a function over time in ARK1-shRYR1 vs shCtrl cells (B) or dantrolene- vs vehicle-treated cells (C). D-F, Mitochondrial ATP and total ATP production rate (basic, pmol/min) in ARK1- shRYR1 or -shCtrl cells (D), cells treated with dantrolene or the vehicle (E), and ARK1-RYR1 or -Control cells (F). G, I, J, L, Relative ATP levels in ARK1 shRYR1 vs shCtrl cells (G), USC

RYR1 vs control cells (I), indicated cells incubated with dantrolene or vehicle (J). Bar charts showing relative ATP production ($\mu\text{mol/g}$) in mouse tumor tissues derived from ARK1-shRYR1 or -shCtrl cells (L). H, K, Mitochondrial membrane potential of USC cells transfected with shRYR1 or shCtrl (H), treated with 50 μM dantrolene or vehicle (K) examined by JC-1 distribution. Green: JC-1 monomer, Red: JC-1 aggregates. M-N, Relative NAD^+/NADH ratios in USC cells transfected with shRYR1 or shCtrl (M) and treated with dantrolene or the vehicle (N). O-P, Relative total and mitochondrial ROS in USC cells transfected with shRYR1 or shCtrl (O) and treated with dantrolene or vehicle (P). In B-P, N = 3 independent experiments. Data represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s., no significance (Student t-test).

Figure 6

RYR1 silencing suppresses TCA cycle and glycolysis. A-B, Heatmaps showing relative levels ($-\log_2$) of differential metabolites in glycolysis and TCA cycle in cell lysates (ARK1-shCtrl vs ARK1-shR1YR1, A; ARK1-vehicle vs ARK1-dantrolene, B) subjected to LC-MS for metabolite measurement. C, Graphic illustration of intermediate metabolites involved in glycolysis and TCA cycle. Green-labeled indicate the decreased ones in RYR1-silenced or dantrolene-treated cells than controls. D, Representative patterns of ECAR from ARK1

cells transfected with shRyR1 or shCtrl. E-F, Relative glutamine levels in USC cells transfected with RyR1-shRNAs or shCtrl (E) and treated with 50 μ M dantrolene or vehicle (F). G, Relative glutamine levels in USC cells transfected with RyR1 full-length cDNA or treated with dantrolene or vehicle (F). In D- G, data represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Student t-test).

Figure 7

Ca²⁺ / AKT/CREB/PGC-1α axis is essential for tumor progression. A-B, PGC-1α mRNA expression in the indicated cells with shCtrl or shRYY1 (A) or incubated with dantrolene or vehicle (B). C, F, Western blot of PGC-1α (C), proteins in AKT/CREB pathway (F) in control- or RYY1-silenced and vehicle- or dantrolene-treated ARK1 cells. D, mETC genes mRNA expression in PGC-1α-silenced (siPGC-1α) or control (siCtrl) ARK1 cells transfected with RYY1 or control. E, Western blot of HK1 and HK2 in control- or RYY1- silenced ARK1 cells. G, Western blot of PGC-1α in ARK1-Control and ARK1-RYY1 cells treated with of AKT or CREB inhibitor vs vehicle. H, Relative ATP level in control and RYY1-overexpressing cells transfected with siCtrl or siPGC-1α. *p < 0.05, **p < 0.01, ****p < 0.0001 (Student t-test). I, mETC genes mRNA expression in ARK1- Control and ARK1-RYY1 cells in the presence of siPGC-1α, AKT (0.1 μM) or CREB inhibitor (0.1 nM) or controls. J, Cell growth of ARK1-Control and ARK1-RYY1 incubated with AKT or CREB inhibitor. *p < 0.05, **p < 0.01, n.s., no significance (2-way ANOVA). K, Western blot of phosphorylated AKT (S473), phosphorylated CREB (S133), and total AKT and CREB in ARK1-shCtrl and ARK1-shRYY1 cells treated with BAPTA or vehicle. L, Graphic illustration of the molecular mechanism. In A, B, D, I, N = 3 independent experiments. HPRT was internal control. *p < 0.05, **p < 0.01, ****p < 0.0001, n.s., no significance (Student t-test). In C, E, F, G, K, N = 3 independent experiments. Data are shown as mean ± SEM. ImageJ software was applied to quantify signals. β-actin was used as loading control.

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