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TITLE: Ryanodine Receptor-1-Dependent Calcium Signaling Pathway as a Novel Tumorigenic Mechanism and Therapeutic Target of Ovarian Cancer

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CONTRACTING ORGANIZATION: Johns Hopkins University, Baltimore, MD

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13. SUPPLEMENTARY NOTES

14. ABSTRACT This award tests the hypothesis that ryanodine receptor-1 (RYR1) upregulation in high grade serous ovarian cancer (HGSOC) represents an aberrant shift in the modality of Ca^{2+} signaling, which contributes to the changes in cellular metabolism and signal transduction, leading to the malignant phenotype of proliferation, migration and invasion; RYR1 over-expression is associated with poor patient survival, and disruption of this mechanism can be effective for ovarian cancer treatment. In this funding period, we completed experiments in several major goals and found that (1) RYR1 overexpression in OVCA432 and OVCA433 cells caused significant increase in resting [Ca^{2+}] compared to the control HOSE cells, indicative of altered Ca^{2+} homeostasis, (2) activation of RYR receptor induced Ca^{2+} release was enhanced in HGSOC cells, (3) RYR1 upregulation is associated with down-regulation of IP_3R expression and suppression of IP_3R-dependent Ca^{2+} release signals, indicative of a switch of modality of Ca^{2+} signaling from IP_3Rs to RYRs, (4) RYR1-dependent store-operated Ca^{2+} entry is enhanced in HGSOC cells, (5) the enhanced proliferation and migration in HGSOC cells were suppressed, while apoptosis was enhanced by shRNA knockdown of RYR1 and the RYR1 antagonist dantrolene, (6) dantolene treatment significantly suppressed OC development in the athymic nude mouse model.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION

Ovarian cancer (OC) is the most common and deadly gynecological malignance worldwide. It accounts 20,000 new cases and over 16,000 deaths per year in the United States. OC is notable for initial chemotherapy sensitivity (>75% response rates) using a combination of platinum and taxane chemotherapy following debulking surgery. However, for the vast majority of patients, OC tumors recur within 12 to 24 months and patients will die of progressively chemotherapy-resistant metastasis. Hence, it is crucial for identifying new therapeutic targets and developing novel therapeutic strategy to prolong patient survival. We approach this problem by analyzing RNAseq data from the Cancer Genome Atlas (TCGA) database to identify genes that are associated with overall survival in patients with advanced stage high-grade serous ovarian cancer (HGSOC), and can be targeted by FDA-approved drugs. We found that the over-expression of the Ca^{2+} release channel ryanodine receptor 1 (RYR1), the target of the FDA-approved drug dantrolene, is associated with poor overall survival in HGSOC patients. The prognostic significance of RYR1 was confirmed by an independent set of microdissected HGSOC samples from MD Anderson Cancer Center. Moreover, RYR1 expression is markedly higher in multiple HGSOC cell lines compared to normal human ovarian surface epithelial cells (HOSE) and fallopian tube epithelial (FTE) cells. Our preliminary studies also showed that RYR1 upregulation alters Ca^{2+} homeostasis in HGSOC, and RYR1-gated Ca^{2+} release is highly effective for endoplasmic reticulum (ER)-mitochondrial Ca^{2+} transfer in HGSOC cells. Inhibition of RYR1 or silencing RYR1 inhibited the expression of the mitochondrial electron transport chain enzymes, and ATP production. Inhibition of RYR1 also suppressed proliferation and motility, and enhanced apoptosis of HGSOC cells. Since IP_3Rs are the predominant Ca^{2+} release channels in normal ovarian and fallopian tube epithelial cells, where RYR1 is minimally expressed, RYR1 upregulation in HGSOC cells may operate as a pathological change of Ca^{2+} signaling in OC. Based on these observations, we proposed the novel hypothesis that RYR1 upregulation in HGSOC represents an aberrant shift in the modality of Ca^{2+} signaling, which contributes to the changes in cellular metabolism and signal transduction, leading to the malignant phenotype of proliferation, migration and invasion; RYR1 over-expression is associated with poor patient survival, and disruption of this mechanism can be effective for ovarian cancer treatment.

2. KEYWORDS

Ovarian cancer, ryanodine receptor 1, Ca^{2+} signaling, endoplasmic reticulum-mitochondrial coupling, dantrolene

3. ACCOMPLISHMENTS

a. What were the major goals of the project?

Major Goal 1: Determine the functional characteristics of RYR1-dependent Ca^{2+} signals in HGSOC cells. (Month 1-6)

Major Goal 2: Determine the functional properties of RYR1-depndent ER-mito coupling in HGSOC cells. (Month 7-12)

Major Goal 3: Determine the contribution of RYR1gated Ca^{2+} release in proliferation, migration, and apoptosis of HGSOC cells. (Month 7-12)

Major Goal 4: Determine whether RYR1-mediated Ca^{2+} release and ROS facilitate PI3K/Akt/mTOR activity in HGSOC cells. (Month 13-24)

Major Goal 5: Determine the mechanisms for the RYR1-dependent regulation of mitochondrial bioenergetics. (Month 25-30)

Major Goal 6: Determine the roles of RYR1 in cancer metastasis and survival in ovarian cancer bearing mice. (Month 1-12)

Major Goal 7: Determine the efficacy of targeting RYR1 for OC treatment. (Month 13-24)

Major Goal 8: Determine whether RYR1 inhibition improve the efficacy of chemotherapy for OC treatment. (Month 25-36)

b. What was accomplished under these goals?

In this funding period, we have performed most of the experiments in *Major Goal 1* for determining the functional characteristics of RYR1-dependent Ca^{2+} signals in HGSOC cells. First, to evaluate the functional roles of RYR1, we determined whether RYR1 overexpression alters Ca^{2+} homeostasis in HGSOC cells. Resting $[Ca^{2+}]_i$ of OVCA432 and OVCA433, and the control HOSE

cells were determined by using the ratiometric Ca^{2+} fluorescent dye Fura-2. Resting $[Ca^{2+}]_i$ of OVCA432 and OVCA433, 87.0±1.8 (n=252) and 86.4±1.9 nM (n=209) respectively, were significantly higher than that of the control HOSE cells (50.8±2.5 nM, n=69, P<0.05)(***Figure 1A***). shRNA knockdown of RYR1 significantly reduced the resting $[Ca^{2+}]_i$ of the RYR1-shRNA transfected OVCA432 cells to the level of control HOSE cells; and overexpression of RYR1 further augmented the resting $[Ca^{2+}]_i$ of OVCA432.

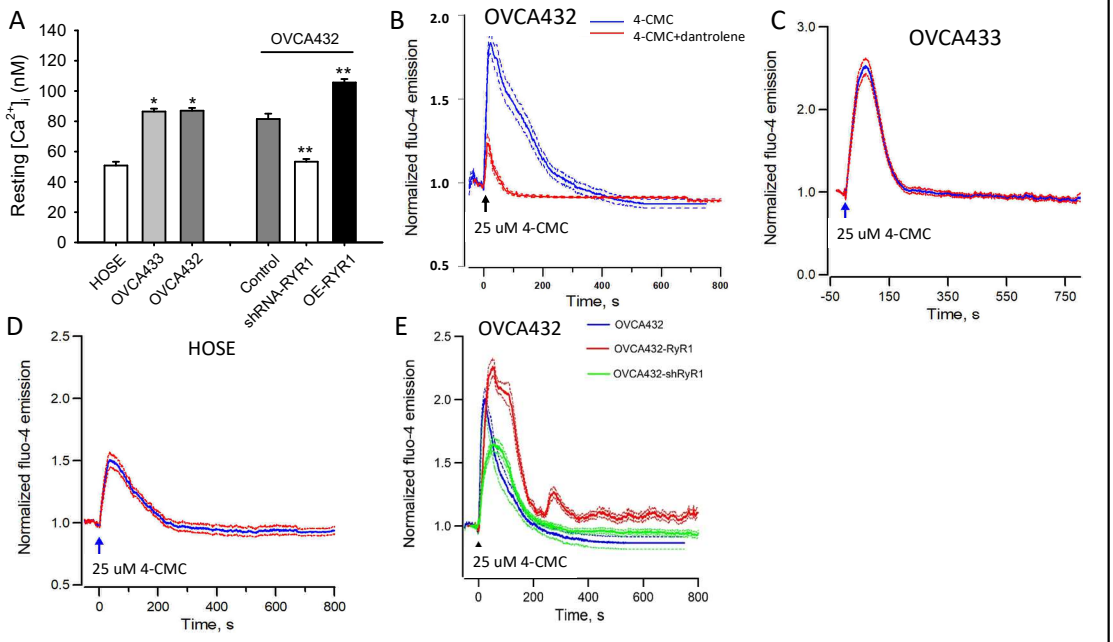

Figure 1 (A) Resting $[Ca^{2+}]$ of OVCA432, OVCA433 and HOSE cells, and OVCA432 cells transfected with shRNA against RYR1 and plasmid for RYR1 overexpression. (B) Ensemble Ca^{2+} transients activated by 25μM 4-CMC in the absence and presence of dantrolene in OVCA432 cells. (C and D) Ca^{2+} transients activated by 25μM 4-CMC in OVCA433 and HOSE cells. (E) Ca^{2+} transients activated by 4-CMC in control and OVCA432 cells transfected with shRNA against RYR1 and a RYR1 plasmid for over-expression.

Second, RYR-dependent Ca^{2+} release was examined by confocal fluorescent microscopy in OVCA432, OVCA433, and HOSE cells loaded with Fluo-4 AM. Application of the common RYR agonist 4-chloro-m-cresol (4-cmc, 25 μM) to OVCA432 cells elicited rapid and large increase in $[Ca^{2+}]_i$, which was blocked after preincubation with the RYR1 antagonist dantrolene (100 μM), confirming the Ca^{2+} response was mediated via the activation of RyRs (***Figure 1B***). 4-cmc also induced robust Ca^{2+} response in OVCA433 cells with peak Ca^{2+} response (***Figure 1C***). In contrast to HGSOC cells, the 4-cmc induced Ca^{2+} response in HOSE cells was significantly smaller, compared to those of OVCA432 and OVCA433 cells (***Figure 1D***). Moreover, silencing RYR1 with shRNA in OVCA432 cells significantly reduced the 4-cmc induced-Ca^{2+} release to a level comparable to that observed in HOSE cells (***Figure 1E***). Overexpression of RYR1 in OVCA432 cells further enhanced the 4-cmc activated Ca^{2+} release. These results clearly suggested that RYR1 upregulation in HGSOC cells is associated with enhanced RYR1-dependent Ca^{2+} release, and altered Ca^{2+} homeostasis in HGSOC cells.

Third, we compared RYR1 and IP_3R-gated Ca^{2+} signaling in HGSOC cells. In non-excitable cells, Ca^{2+} release from ER is gated predominantly by IP_3Rs, and RyRs are minimally expressed. RYR1 upregulation in HGSOC may suggest a shift in the ER Ca^{2+} release and Ca^{2+} signaling processes in HGSOC cells. To examine this possibility, the correlation of RYR1 and IP_3R expression was analyzed in OC cells microdissected from patient samples. ddPCR analysis showed that the expression of RYR1 in individual OC samples was negatively correlated between RYR1 and IP3R1 (R=-0.463, P=0.0173, n=27) and between RYR1 and IP3R2 (R=-0.507, P=0.0083, n=27) (***Figure 2A***). Analysis of RYR1 and IP_3Rs expression in OVCA432 cells also showed that RYR1 over-expression was associated with significant reduction in expression of IP_3R1, IP_3R2 and IP_3R3 (***Figure 2B***), whereas shRNA knockdown RYR1 led to significant increase in IP_3R2 and IP_3R3

expression (data not shown). The Ca^{2+} signals of IP_3R and RyR-gated Ca^{2+} release were compared in OVCA432, OVCA433, and the HOSE cells. Activation of IP_3R in OVCA432 cells by the cell-permeant IP_3 analogue $Bt_3(1,3,5)IP_3$/AM (200 μM) elicited cytosolic Ca^{2+} oscillations, which was significantly suppressed by preincubation with the IP_3R antagonist xestospongin C (10 μM) for 15 minutes (***Figure 2C***). Similar IP_3-induced Ca^{2+} oscillations were observed in OVCA433 cells (data no shown). In contrast, IP_3-AM induced a robust phasic Ca^{2+} response without apparent Ca^{2+} oscillation in the control HOSE cells (***Figure 2D***), where the peak Ca^{2+} response was significantly higher than those of OVCA432 and OVCA433 cells. The down-regulation of IP_3Rs and their Ca^{2+} release signals associated with RYR1 upregulation in HGSOC cells suggested that there is a major shift in modality of Ca^{2+} signaling in HGSOC cells during OC development.

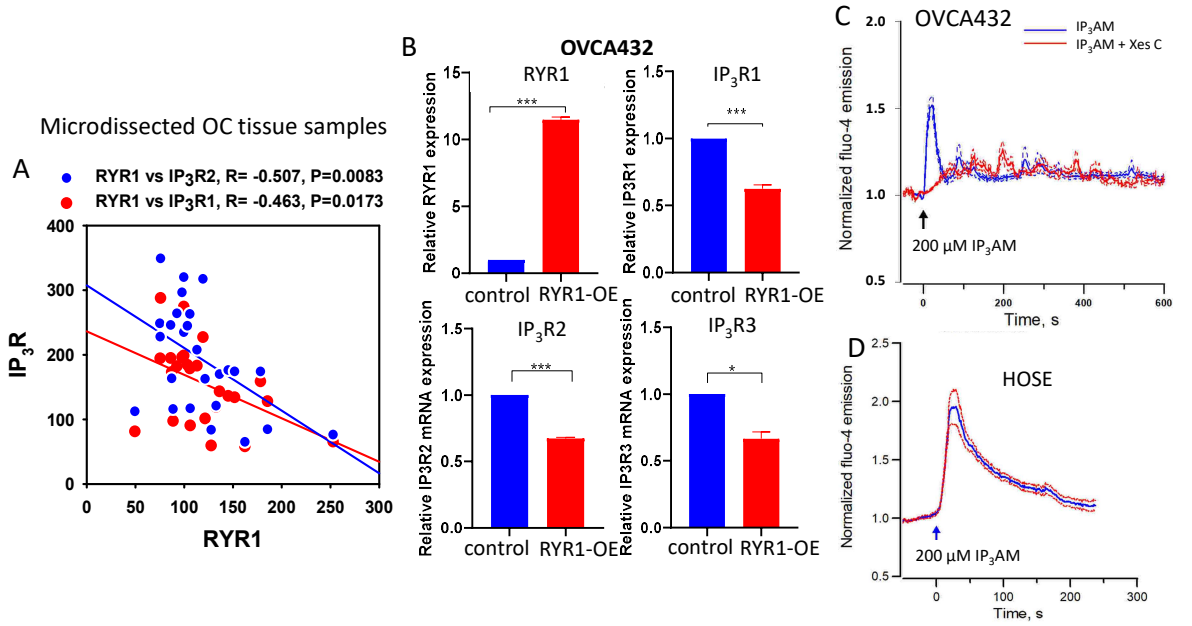

Figure 2 (A) Correlation analysis of RYR1 and IP_3R expression in individual OC tumor samples from patients (n=27). (B) RYR1 and IP_3R1, 2 and 3 expression in control and RYR1 over-expressed OVCA432 cells. (C) Oscillatory Ca^{2+} transients activated by 100 μM of membrane permeant IP_3-AM in the absence or presence of the IP_3R antagonist xestospongin C in OVCA432 cells. (D) Robust phasic Ca^{2+} transients activated by 100 μM of IP_3-AM in HOSE cells.

Fourth, we have characterized RYR1-dependent store-operated Ca^{2+} entry (SOCE) in HGSOC cells. SOCE in OVCA432 and HOSE cells were activated by depleting ER Ca^{2+} store with the SERCA inhibitor thapsigargin (10 μM) in Ca^{2+}-free external solution. Reintroduction of 2 mM Ca^{2+} in the external solution elicited a large Ca^{2+} influx signal, which was blocked by the non-selective SOCE blocker SKF-96365 (30 μM). Thapsigargin-induced SOCE in OVCA432 cells was significantly higher than that of the HOSE cells (***Figure 3A,B***), indicative of enhanced SOCE in OVCA432 cells. RYR-dependent SOCE was examined by preincubating cells with 4-cmc (25 μM) to deplete RYR-gated Ca^{2+} stores. Similar to thapsigargin, 4-cmc-induced SOCE was significantly higher in OVCA432 cells compared to that in the HOSE cells (***Figure 3C,D***). It was completely blocked by the selective SOCE antagonist GSK7975A, and suppressed by RYR1-shRNA in OVCA432 cells (***Figure 3E***). These results clearly suggested that RYR1 is capable of mediating SOCE, and its upregulation contributed to the enhanced SOCE in HGSOC cells.

We have also completed most of the experiments in *Major Goal 3* for determining the contribution of RYR1gated Ca^{2+} release in proliferation, migration, and apoptosis of HGSOC cells. Transfection of OVCA432 cells with two different sequences of shRNA both cause significant reduction in mRNA and protein compared to the control shRNA sequence, and RYR1 overexpression caused significant increase in RYR1-mRNA and protein (***Figure 4A***). Neither RYR1-shRNA transfection nor RYR1-overexpression altered RYR2 and RYR3 expression (data not shown). shRNA silencing of RYR1 caused clear reduction in OVCA432 proliferation, whereas RYR1-overexpression enhanced proliferation measured by MTT assay (***Figure 4B and C***). Inhibition of RYR activity with the FDA-

approved RYR1 antagonist dantrolene (50 or 100 μM) caused approximately 50% reduction in OVCA432 cells measured by the MTT assay, but it had only minor effect on the control HOSE cells where RYR1 expression were minimal (***Figure 4D***). It is noteworthy that 100 μM dantrolene failed to inhibit proliferation of OVCA432 cells transfected with RYR1-shRNA (***Figure 4E***), suggesting the suppressive effect of dantrolene on OC cell proliferation was specific to RYR1 inhibition. Moreover, RYR1-shRNA significantly inhibited OVCA432 migration measured by the Transwell assay, and slightly enhanced the apoptosis ratio determined by flow-cytometry using annexin V-FITC and PI (***Figure 4F and G***). Dantrolene at 25-100 μM caused concentration-dependent inhibition of migration in OVCA433, and OVCA90 cells (***Figure 4F***). Dantrolene (100 μM) also increased the apoptosis ratio of OVCA432, OVCA433, and OV90 cells (***Figure 4G***). These results clearly suggested that RyR1 upregulation contributes to proliferation, migration, and anti-apoptosis; inhibition of RYR1 expression or its activity suppressed these malignant phenotypes of HFSOC cells.

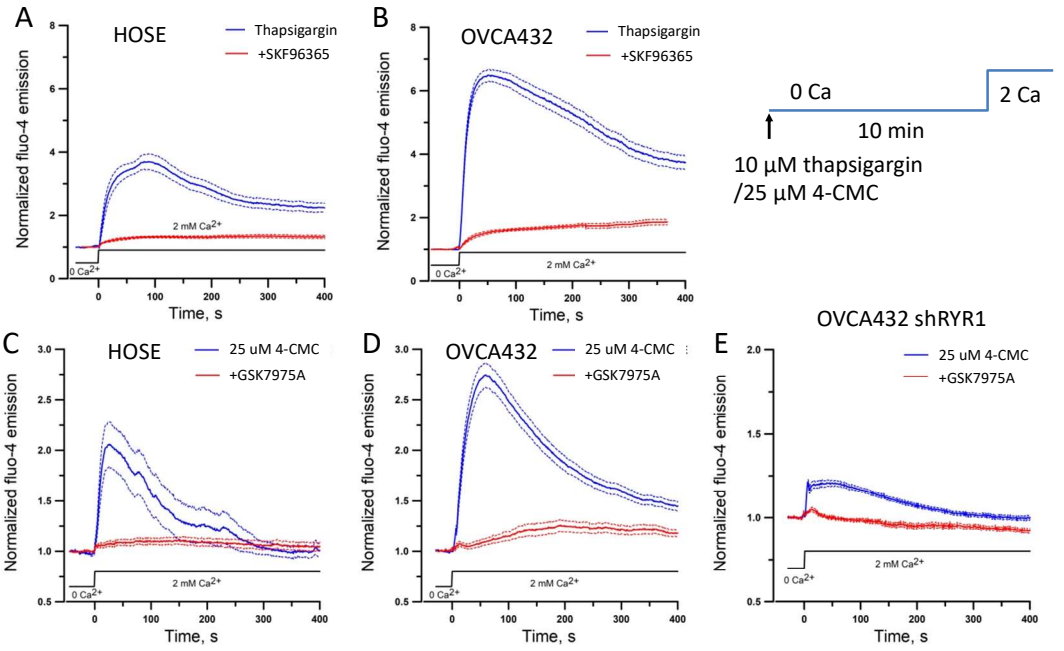

Figure 3 (A and B) Store-operated Ca^{2+} entry (SOCE) activated by pretreatment of 10 μM thapsigargin in the absence or presence of the SOCE antagonist SKF96365 in the control HOSE cells and OVCA432 cells. (C, D, and E) RYR-dependent SOCE activated by pretreatment 25 μM 4-cmc in the absence or presence of the SOCE antagonist GSK7975A in the control HOSE cells, OVCA432 cells and OVCA432 transfected with RYR1 shRNA.

We have completed part of the experiments in ***Major Goal 7*** for determining the efficacy of targeting RYR1 for OC treatment. An ovarian cancer mouse model was generated by intra-ovary injection of luciferease-labeled HGSOC cells into athymic nude mice. 4 weeks after OVCA432 cells injection, dantrolene (5 mg/kg, i.p) was given every other day for 5 weeks (***Figure 5A***). The results showed that dantrolene treatment caused significant reduction in tumor mass evaluated by bilumininescence (n=10 each group, P<0.019) and by tumor weight post-mortem (P<0.001)(***Figure 4B, C***). Immunohistocytochemistry also showed significant reduction in the proliferative cells positive of Ki67 in the tumor tissue of the mice (***Figure D, E***). These results raise the intriguing possibility that RYR1 can be considered as a target for the treatment of HGSOC. We are also performing the experiments in ***Major Goal 6*** for determining the role of RYR1 in cancer metastasis in ovarian cancer bearing mice. Luciferease-labeled OVCA432 cells transfected with control and shRNA against RYR1 were injected into athymic nude mice. The treatment has been completed, and we are in the process of evaluating the progression of OC development.

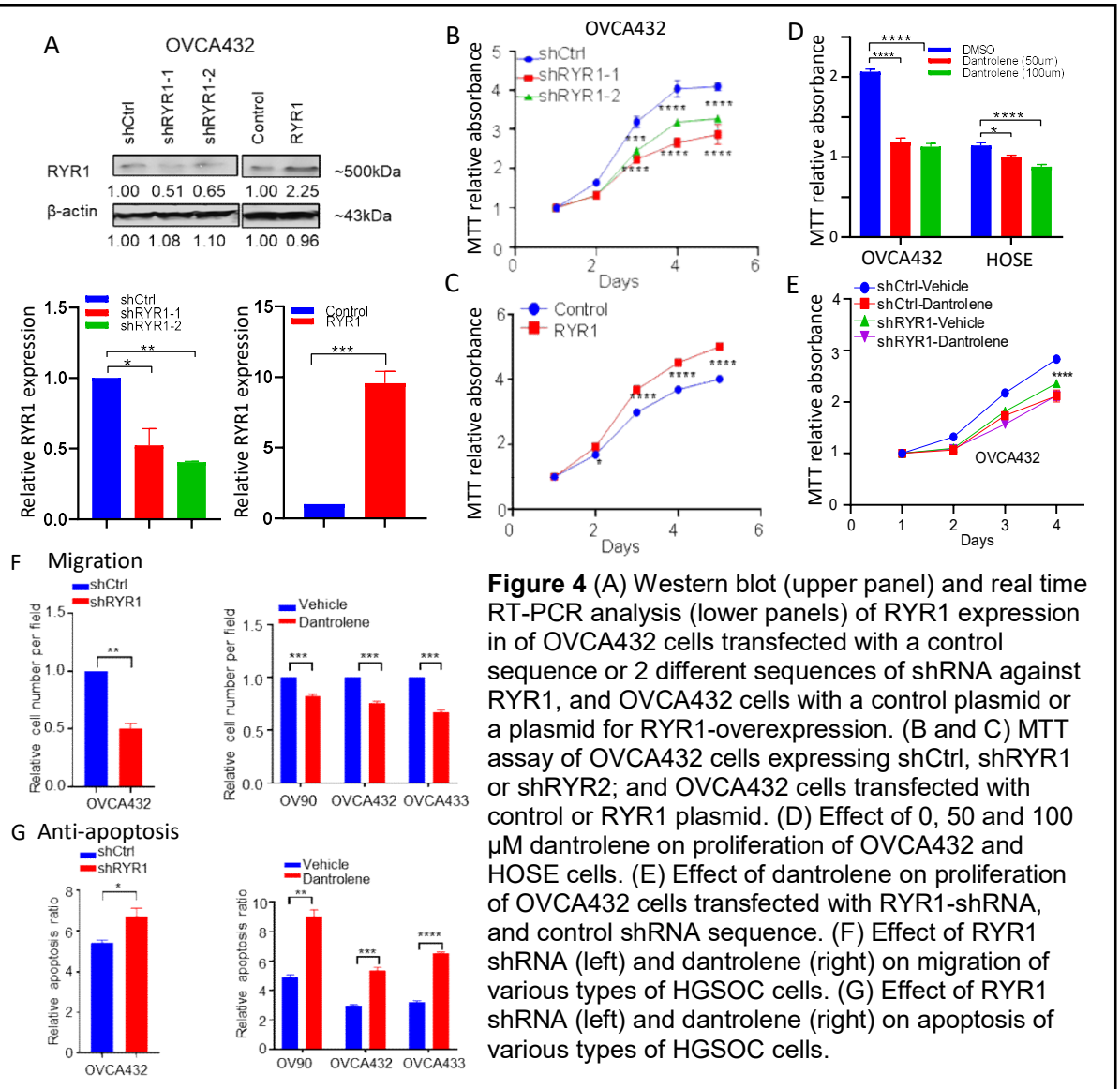

Figure 4 (A) Western blot (upper panel) and real time RT-PCR analysis (lower panels) of RYR1 expression in of OVCA432 cells transfected with a control sequence or 2 different sequences of shRNA against RYR1, and OVCA432 cells with a control plasmid or a plasmid for RYR1-overexpression. (B and C) MTT assay of OVCA432 cells expressing shCtrl, shRYR1 or shRYR2; and OVCA432 cells transfected with control or RYR1 plasmid. (D) Effect of 0, 50 and 100 μM dantrolene on proliferation of OVCA432 and HOSE cells. (E) Effect of dantrolene on proliferation of OVCA432 cells transfected with RYR1-shRNA, and control shRNA sequence. (F) Effect of RYR1 shRNA (left) and dantrolene (right) on migration of various types of HGSOC cells. (G) Effect of RYR1 shRNA (left) and dantrolene (right) on apoptosis of various types of HGSOC cells.

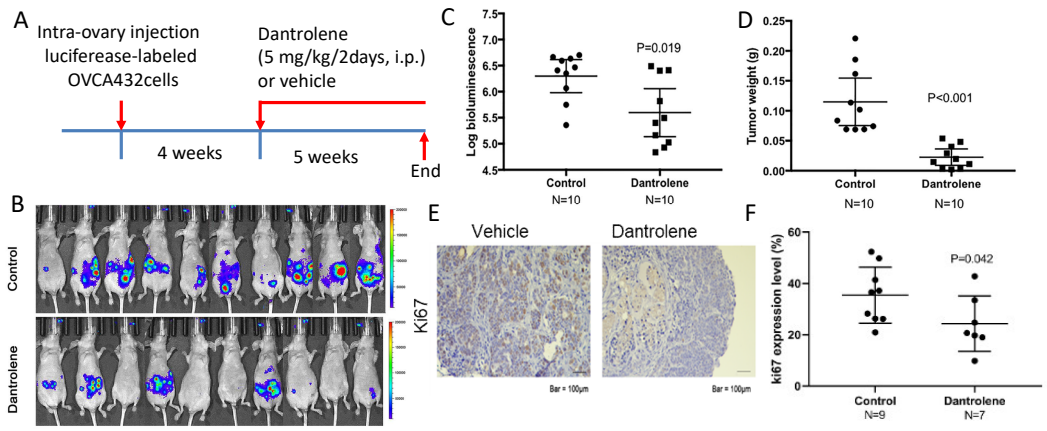

Figure 5 (A) Protocol for the dantrolene treatment OC-induced by intra-ovary injection of luciferase-labeled OVCA432 cells in athymic nude mice. (B) Images of luminescence measured in the OC-bearing mice with or without dantrolene treatment measured at the end of the protocol. (B and C) Significant reduction in bioluminescent signal and tumor weight in dantrolene treated mice. (E and F) Immunohistochemistry analysis of cell the proliferation maker Ki67 in the OC tumor tissue of mice with or without dantrolene treatment.

c. What opportunities for training and professional development has the project provided?
Nothing to report

d. How were the results disseminated to communities of interest?
Nothing to report

e. What do you plan to do during the next reporting period to accomplish the goals?
During the next reporting period (months 13-24), we will perform experiments according to those outlines in the proposal to (1) determine the functional properties of RYR1-depndent ER-mito coupling in HGSOC cells, (2) determine whether RYR1-mediated Ca^{2+} release and ROS facilitate PI3K/Akt/mTOR activity in HGSOC cells; (3) determine the roles of RYR1 in cancer metastasis and survival in ovarian cancer bearing mice, and (4) determine the efficacy of targeting RYR1 for OC treatment.

4. IMPACT

a. What was the impact on the development of the principal disciplines of the project?
Nothing to report

b. What was the impact on other disciplines?
Nothing to report

c. What was the impact on technology transfer?
Nothing to report

d. What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS

Nothing to report

6. PRODUCTS

Kay-Pong Yip, Chi-Lam Au-Yeung, Li-Zhang, Samuel C. Mok, and James S.K. Sham. Upregulation of ryanodine receptor-1 dependent calcium signaling contributes to the malignance of high-grade serous ovarian cancer (HGSOC). Biophysical journal 122 (3), 163a, 2023 (Abstract)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. What individuals have worked on the project?
James S.K. Sham, Ph.D., P.I.
Samuel C. Mok, Ph.D., Multi-P.I.
Kay-Pong Yip, Ph.D., subaward P.I.
Omkar Paudel, technican

b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
No

c. What other organizations were involved as partners?
Organization Name: N/A
Location of Organization: N/A
Partner's contribution to the project: N/A
Financial support: N/A
In-kind support: N/A
Facilities: N/A
Collaboration: N/A
Personnel exchanges: N/A
Other:

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES

Control/Tracking Number: 23-A-2808-BPS
Activity: Abstract
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UPREGULATION OF RYANODINE RECEPTOR-1 DEPENDENT CALCIUM SIGNALING CONTRIBUTES TO THE MALIGNANCE OF HIGH-GRADE SEROUS OVARIAN CANCER (HGSOC)

Author Block: : Kay-Pong D. Yip[1], Chi-Lam Au-Yeung[2], Li Zhang[2], Samuel C. Mok[2], **James S.K. Sham**[3].
[1]University of South Florida, Tampa, FL, USA, [2]MD Anderson Cancer Center, Houston, TX, USA, [3]Dept Med, Johns Hopkins Univ Sch Med, Baltimore, MD, USA.

Abstract:
Ca^{2+} signaling plays crucial roles in cancer metastasis and progression. We analyzed RNAseq data from TCGA database and our patient samples; and identified over-expression of ryanodine receptor 1 (RYR1), the target of the FDA-approved drug dantrolene, is associated with poor survival of HGSOC patients. RYR1 expression is also markedly higher in several HGSOC cell lines compared to normal human ovarian surface epithelial cells (HOSE) and fallopian tube epithelial cells. Suppression of RYR1 expression/activity by shRNA or dantrolene significantly inhibited proliferation and migration of HGSOC cells (OVCA432, OVCA433, OV90). Ca^{2+} fluorescence measurement showed that the resting [Ca^{2+}] and Ca^{2+} release triggered by the RYR-agonist 4-chloro-m-cresol (4-cmc) were significantly higher in HGSOC cells than the HOSE cells. Store-operated Ca^{2+} entry (SOCE) induced by 4-cmc was also enhanced in HGSOC cells. The elevated resting [Ca^{2+}], the 4-cmc-induced Ca^{2+} release and SOCE in the HGSOC cells were reduced to the control level by shRNA against RYR1; and were significantly potentiated by over-expression of RYR1. RYR-dependent endoplasmic reticulum (ER)-mitochondrial Ca^{2+} transfer was examined by expressing the ER specific Ca^{2+} biosensor G-CEPIA1er and mitochondria Ca^{2+} biosensor CEPIA2mt in HGSOC cells. Activation of RYR by 4-cmc triggered a large reduction in ER [Ca^{2+}], which was associated with a robust increase in mitochondrial [Ca^{2+}]. Activation of RYRs by photorelease of caged cADP-ribose in a subcellular region caused an immediate transient local reduction in ER [Ca^{2+}] and a fast transient local increase in mitochondria [Ca^{2+}]. In contrast, RyR-dependent ER-mitochondrial Ca^{2+} transfer was not observed in the HOSE cells. These observations are the first direct evidence of enhanced RYR-mediated ER-mitochondrial Ca^{2+} transfer in ovarian cancer cells, and upregulation of RYR1-dependent Ca^{2+}-signaling pathway contributes to the malignant phenotypes of HGSOC cells.

Presenter Information (Complete):
*** Select Gender:** Male
Asian : True
*** Select Career Level:** Other

Presentation Preference (Complete):
: Platform or Poster
: No

Sponsorship (Complete):
Topic (Complete): 6G Ion Channels, Pharmacology, and Disease ; 5D Calcium Signaling
Technique (Complete):
First Selection: Fluorescence and Light Microscopy
Second Selection: Cell/Tissue Imaging and Mechanics

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