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CONTRACTING ORGANIZATION: Hudson Institute of Medical Research

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14. ABSTRACT

Ovarian granulosa cell tumors (GCT) are hormonally-active neoplasms characterized by endocrine manifestations, an indolent course and late recurrence. Treatment involves surgery, and chemo- or hormonal-therapy have limited efficacy. The hypothesis of this proposal is that 1) PPARgamma and XIAP play fundamental roles in the regulation of granulosa cell (GC) apoptosis, and/or terminal differentiation, albeit in a reciprocal manner, and 2) that combined targeting of PPARgamma and XIAP presents a novel therapeutic strategy for GCT treatment. Our specific aims are to 1) demonstrate the efficacy of PPARgamma activation in combination with XIAP inhibition for the treatment of GCT in a xenograft and 3D cell culture model; 2) elucidate the mechanisms of PPAR□induced apoptosis and/or differentiation through analysis of the PPARgammacistrome and transcriptome in GC- and GCT-derived cells; and 3) to determine the mechanistic consequences of XIAP inhibition and PPAR□activation at the proteomic level in GCT. We have evidence to show in vitro that the combination therapy proposed is efficacious in GCT-derived cell lines and in GCT tissue explants. An investigation of the genes that regulate this response indicate an activation of the NFkappaB

15. SUBJECT TERMS

Ovarian Cancer, Granulosa Cell Tumors, XIAP, PPARgamma, Smac-mimetics

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Background: Ovarian granulosa cell tumors (GCT) are hormonally-active neoplasms characterized by endocrine manifestations, an indolent course and late recurrence. Treatment involves surgery, and chemo- or hormonal-therapy have limited efficacy. This proposal will address the development of a novel GCT-specific therapeutic strategy. The survival transcription factor, NFκB, is activated in these tumors; inhibition of this pathway promotes apoptosis. Peroxisome proliferator-activated receptor-gamma protein (PPARγ), a transcription factor that impedes growth and promotes differentiation, is overexpressed in GCT, but transrepressed by NFκB signalling in GCT. An NFκB-induced protein, X-linked inhibitor of apoptosis protein (XIAP), is also overexpressed in GCT and is critical in preventing GCT cell apoptosis and represents an attractive therapeutic target. We **hypothesize** that 1) PPARγ and XIAP play fundamental roles in the regulation of granulosa cell (GC) apoptosis, and/or terminal differentiation, albeit in a reciprocal manner, and 2) that combined targeting of PPARγ and XIAP presents a novel therapeutic strategy for GCT treatment.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Ovarian Cancer; Granulosa Cell Tumors, X-Linked Inhibitor Of Apoptosis Proteins (XIAP); Peroxisome Proliferator Activated Protein (PPARγ)

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1(specified in proposal)	Timeline	Site 1	Status
Major Task 1: Demonstrate the efficacy of PPARγ activation in combination with XIAP inhibition for the treatment of GCT in a xenograft model.	Months		
Submission of institutional approved animal protocols and related material for DoD's ACURO approval	-2 to -3	SC	Expect approval in Nov 2020 – see Problems
Receive ACURO approval before initiating experiments	0 - 2	SC	
Submission of institution's IRB approval and related material for DoD's HRPO approval	-2 to -3	SC PJF	Approved
Receive HRPO approval before initiating experiments	0 – 2	SC PJF	

Subtask 1: Stable transfection of parental GCT-derived cells with RFP expression vector Appointment of research assistant and part time Postdoc for the *in vitro* studies	1-3	SC	Complete Nov 2018
Subtask 2: Establishment of parental and TET-on GCT-derived cell line xenografts	5-12	SC	Complete July 2019
Subtask 3: Breeding to generate between 100 to 120 NOD/SCID mice for xenograft and/or PDX experiments. Colonies of NOD/SCID mice are established at our Institute. For the xenograft studies we will require approximately 100 to 120 mice. In order to generate this number, we will maintain 6 pairs / year x 7 litters (n=6 pups / litter; 50% female).	5-16	SC	Postponed 2021
Subtask 4: Mice randomized and treated with the treatment regimen outlined in the proposal The *in vivo* animal model proposed in Aim 1.1 requires groups of 6 x nod/SCID mice for each experimental regimen (n=8) for each cell line. This equates to 6 mice x 8 (treatments) x 2 (cell lines) = 96 mice.	7-16	SC PJF JS	Postponed 2021
Subtask 5: xenografts removed for analysis (number, dimension, weight, volume and histology). Protein expression for markers of proliferation; apoptosis; angiogenesis; and the target proteins; XIAP and PPARγ will be examined using immunohistochemistry.	9 – 24	SC	Postponed 2021
Subtask 6: PDX models – dependent on availability of patient tissue from surgery – this will follow a similar regimen as outlined above for the xenograft models. Expected animal numbers: For every PDX sample, we expect to grow in 4 animals with 4 harvests over 3 years = 16 animals	7- 24	SC PJF JS	Postponed 2021
Major Task 2: Demonstrate the efficacy of PPARγ activation in combination with XIAP inhibition for the treatment of GCT in an ex-vivo and 3D cell culture model.			
Subtask 1: Spheroid experiments will commence with treatments	6 - 15	SC JS	Complete Jan 2019
Subtask 2: ex-vivo explant studies of patient material should they be collected during this period	6 - 36	SC PJF	Complete Jan 2019
Subtask 3: Manuscript prepared for xenograft and 3D model findings	24 -	SC PJF	3D Model Published 2019

Specific Aim 2 (specified in proposal)	Timeline	Site 1	Status
Major Task 3: To elucidate the mechanisms of PPARγ induced apoptosis and/or differentiation through analysis of the PPARγ cistrome in GC- and GCT-derived cells.			
Subtask 1: Treatment of cell lines will begin, and chromatin prepared for ChIP-Seq (Aim 2.1).	1 - 6	SC	Complete March 2020
Subtask 2: Perform ChIP-Seq			Samples to be submitted Nov 2020
Subtask 3: ChIP-Seq Bioinformatic analysis using specific pipelines outlined (Aim 2.1). Differentially expressed genes will be identified and the best candidates chosen for characterization (Aim 2.3).	12 - 18	SC RF	To be performed 2021
Major Task 4: To elucidate the mechanisms of PPARγ induced apoptosis and/or differentiation through analysis of the PPARγ transcriptome in GC- and GCT-derived cells.			
Subtask 1: Treatment of cell lines and RNA prepared for RNA-Seq (Aim 2.2).	6-12	SC RF	Completed October 2020
Subtask 2: Perform RNA-Seq	1-6	SC	Samples submitted October 2020
Subtask 3: RNA-Seq Bioinformatic analysis using specific pipelines outlined (Aim 2.2). Differentially expressed genes will be identified and the best candidates chosen for characterization (Aim 2.3).	6-9	SC	Results received Mar 2020 – in progress
Major Task 5: Determining the functional significance of PPARγ-regulated genes			
Subtask 1: Submission of ethics proposals to institutional animal ethics committee	12 – 13	SC	Submission Nov 2020
Subtask 2: Submission of institutional approved animal protocols and related material for DoD's ACURO approval	14 – 15	SC	
Subtask 3: Receive ACURO approval before initiating experiments	16 - 17	SC	
Subtask 4: Characterization of PPARγ-regulated genes will commence (Aim 2.3 (a), (b) and (c)).	18-24	SC RF PJF	In Progress
Subtask 5: Knockdown or re-expressed expression in cells (Aim 2.3c).	24 - 30	SC	In Progress

Subtask 6: Assessment for proliferation, viability, apoptosis, invasiveness, transactivation assays, colony formation assays and xenografts in gene knock down or re-expression in GC- and GCT-derived cell lines. Estimated animal numbers for xenograft experiments: groups of 6 x nod/SCID mice for each cell line with either knock-down or overexpression of candidate genes identified (n=5),for each cell line ie 6 mice x 5 (candidates) x 2 (cell lines) = 60 mice.	26 - 36	SC PJF	In Progress
Subtask 7: Publication preparation for Aim 2	30 - 36	SC RF JS PJF	Aim to submit July 2021
Major Task 6: To determine the mechanistic consequences of XIAP inhibition and PPARγ activation at the proteomic level in GCT.			
Subtask 1: Transduction experiments will begin with wild type and mutant XIAP expression vectors re-expressed (n=5 expression constructs) in XIAP-deficient cell lines (Aim 3.1).	1 - 6	SC	Complete March 2019
Subtask 2: SILAC experiments commenced using cell lines with XIAP re-expression (Aim 3.1).	6-18	SC JS AS	In Progress
Subtask 3: Aim 3.1 will have been completed. Bioinformatic analysis completed. Aim 3.2 characterization of top 2 candidate proteins will have commenced.	12 - 24	SC JS AS	In Progress
Subtask 4: Integration of SILAC data, ChIP-Seq data and RNA-Seq data will be performed.	24 - 36	SC RF AS JS PJF	In Progress
Subtask 5: Publication of Aim 2 and 3 findings completed	36	SC RF JS AS PJF	Aim to submit Dec 2021

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Specific Aims: 1. To demonstrate the efficacy of PPARγ activation in combination with XIAP inhibition for the treatment of GCT in a xenograft and 3D cell culture model; **2.** To elucidate the mechanisms of PPARγ induced apoptosis and/or differentiation through analysis of the PPARγ cistrome and transcriptome in GC- and GCT-derived cells; and **3.** To determine the mechanistic consequences of XIAP inhibition and PPARγ activation at the proteomic level in GCT.

Update on Progress:

Aim 1. To demonstrate the efficacy of PPARγ activation in combination with XIAP inhibition for the treatment of GCT in a xenograft and 3D cell culture model.

Rationale

Targeting more than one pathway in cancer is known to be more efficacious than targeting a single pathway (Fulda and Vucic 2012). Building on our *in vitro* findings using monolayer cultures, we will show that PPARγ activation and XIAP inhibition abrogates tumor development using: (i) an *in vivo* murine xenograft preclinical model; and (ii) an *ex vivo* explant and an established 3D cell culture model that mimics the tumor microenvironment.

Results:

Aim 1.2: Completed – see Year 1 Report

Updated results for combined XIAP inhibition and PPAR activation in primary patient-derived GCT explants:

In our first report, we showed that combination treatment also disrupted 2 separate GCT explants after 7-day treatment, with concomitant loss of cell viability. We have further shown that this is also true for a further 2 recurrent GCT explant samples performed during the year when surgical material became available.

Works In Progress:

Aim 1.1: Develop an *in vivo* xenograft model

This in vivo work outlined in the grant has been delayed due to a number of reasons. The primary delay has been the animal facility located at our Institute has encountered issues centering on construction works of a nearby building that has affecting the breeding pattern of animals. The difficulties of performing our work at the Hudson Animal Facility has been a source of frustration, and have caused a delay in my seeking ethics until I am sure I can perform the experiments in a suitable facility with minimal likelihood in interruptions to the experiments. Hence, I have only just begun to initiate animal ethics applications that will now utilise the animal facilities at nearby Monash University in collaboration with a researcher at the Biomedical Discovery Institute at Monash University. Unfortunately, also, COVID-19 has also become an interruption to this, as currently our State has experienced considerable lockdown periods that have disrupted lab operations since April 2020. We are hoping that our laboratory work will be allowed to restart within the next month. This has also had implications regarding applications for animal ethics, as the committees are meeting irregularly, and hence significant delays in assessing and finalising applications.

I anticipate that we will be able to receive our animal ethics approvals by the end of the year, and will be able to commence our animal studies in the very first week of 2021.

Aim 2: To elucidate the mechanisms of PPARγ induced apoptosis and/or differentiation through analysis of the PPARγ cistrome and transcriptome in GC- and GCT-derived cells.

Rationale

We hypothesize that understanding the mechanisms of PPARγ activation in GCT will (i) identify potential response molecular markers to monitor how GCT will respond to this novel therapy, and (ii) potentially provide prognostic information on GCT response to therapy. PPARγ is a transcription factor that acts primarily by regulating gene transcription. Hence, the GCT-derived cell lines provide a unique model to fully characterize the consequence of this ligand-dependent transcription factor in ovarian biology and disease. To test this, we will draw a complete high-resolution binding map across the GC genome using ChIP-Seq, combined with whole transcriptome data obtained from RNA-Seq, to provide an unprecedented level of resolution for PPARγ activation in this cell type.

Aim 2.1: To investigate PPARγ DNA interactions at a genome-wide level

Works in Progress:

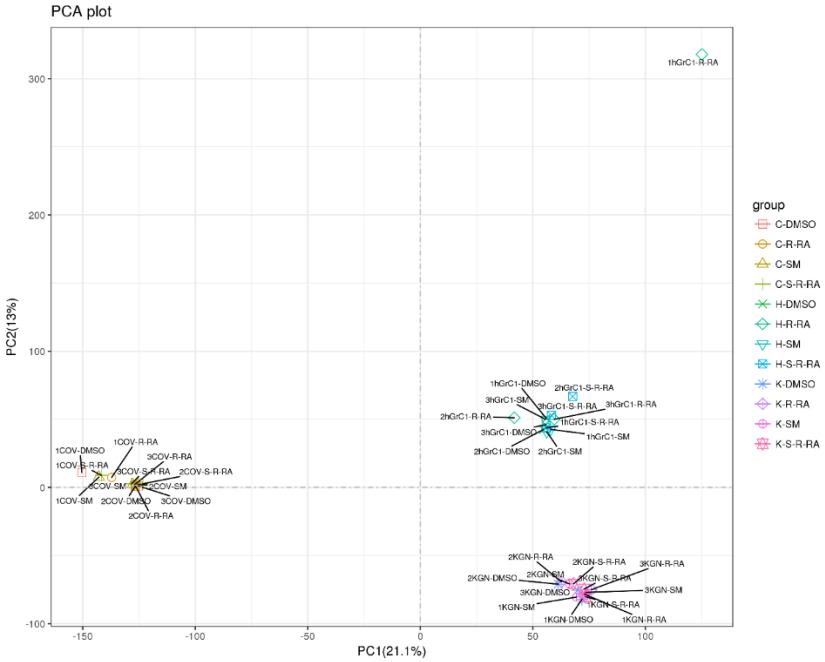
We have processed all samples in order to perform ChIP-Seq. The samples were ready to go to the next stage of library preparation, however, as a result of our state's COVID-19 lockdown, there has been a delay in getting these samples to the genomics facility for generation of the libraries and subsequent sequencing. We anticipate submission of these samples now from the week of 12^{h} October 2020.

Aim 2.2: Whole transcriptome analysis by RNA-Seq

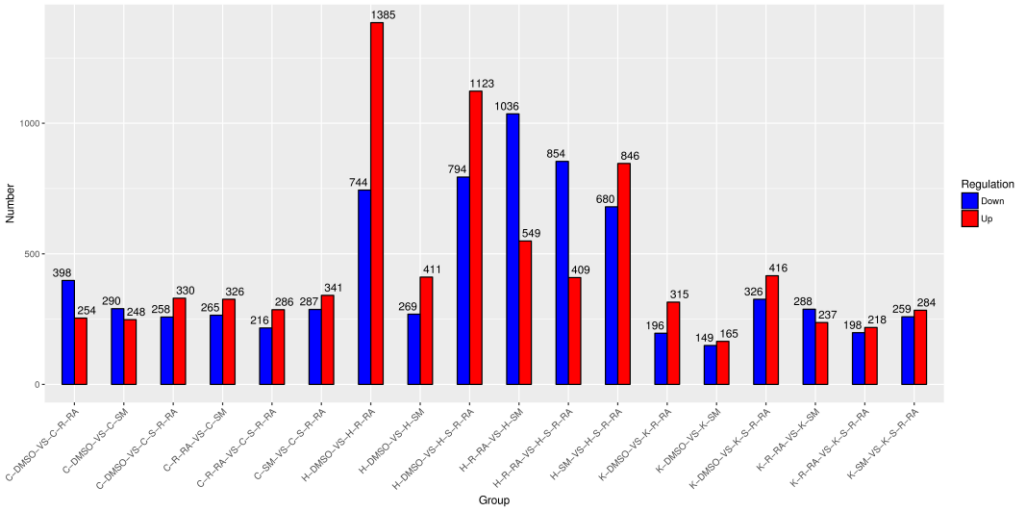
We have confirmed functional PPARγ target genes in the KGN, COV434, and hGrC1 cell lines by RNA-Seq analyses. Cells were treated with either: vehicle, Smac mimetic alone (XIAP inhibition), or RGZ/RA (PPARγ/ RXRα activation) with and without Smac mimetic for 4 hours. We are currently analyzing the RNA-Seq data where reads have been mapped against the reference genome (GRCH38.p2). Principal component analysis (PCA) shows close clustering of each individual cell line, indicating appreciable agreement of the RNA-Seq quality control (**Fig. 1a**), with the exception of one replicate (1hGrC1-Rgz/RA) which has been removed from the analysis. Preliminary data shows a significant number of genes induced by the PPARγ and RXRα ligands in the KGN, COV434 and hGrC1 cells (**Fig. 1b**). Venn diagrams (**Fig. 1c**), are presented showing some of the most significantly regulated genes (> 5 fold; FDR 0.001), showing some uniquely regulated PPARγ/RXRα genes, and also that for all three cell lines, Smac-mimetics induce genes involved in the non-canonical NFκB pathway. We are currently validating some of the top regulated genes, performing expression profiles in the treated cells as well GCT explants that have been treated with the compounds. We are also identifying genes that are targetable using small molecule inhibitors, and which we will add to a high-throughput compound small molecule library screen containing a panel of 2300 approved or in advanced staged clinical trials. The functional significance of these genes are also being planned using CRISPR to knock out the function of overexpressed genes, and re-expression using lentiviral transduction. The functional consequences will be evaluated with respect to proliferation, viability, apoptosis, invasiveness, colony formation, and apoptosis. Animal ethics are also being written with respect to using candidate gene knock-out or overexpressed KGN and COV434 cells in a xenograft model.

Figure 1

A.


PCA plot
PC2(13%)
PC1(21.1%)
group
C-DMSO
C-R-RA
C-SM
C-S-R-RA
H-DMSO
H-R-RA
H-SM
H-S-R-RA
K-DMSO
K-R-RA
K-SM
K-S-R-RA

B.


Number
Group
Regulation
Down
Up
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C-DMSO-VS-C-SM
C-DMSO-VS-C-S-R-RA
C-R-RA-VS-C-SM
C-R-RA-VS-C-S-R-RA
C-SM-VS-C-S-R-RA
H-DMSO-VS-H-R-RA
H-DMSO-VS-H-SM
H-DMSO-VS-H-S-R-RA
H-R-RA-VS-H-SM
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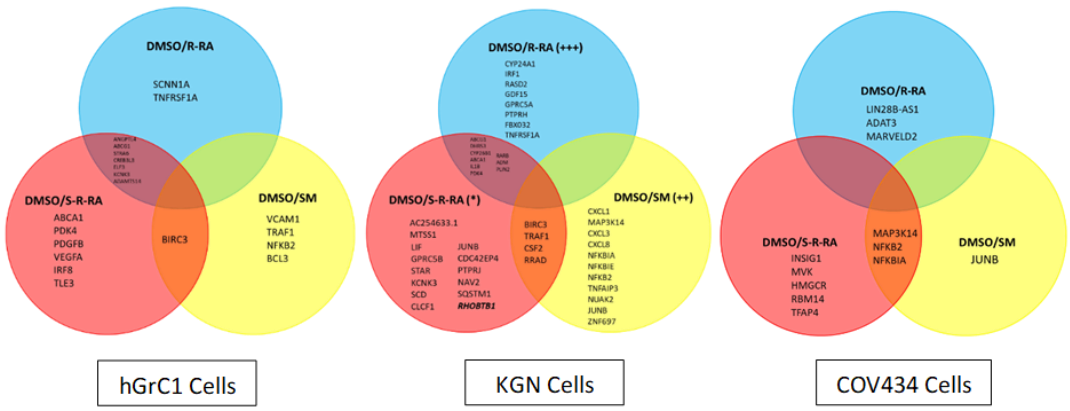
K-SM-VS-K-S-R-RA

C.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SCNN1A
TNFRSF1A
DMSO/S-R-RA
ABCA1
PDK4
PDGFB
VEGFA
IRF8
TLE3
BIRC3
DMSO/SM
VCAM1
TRAF1
NFKB2
BCL3
hGrC1 Cells
DMSO/R-RA (+++)
CYP24A1
IRF1
RASD2
GDF15
GPRC5A
PTPRH
FBXO32
TNFRSF1A
DMSO/S-R-RA (*)
AC254633.1
MTSS1
LIF
GPRC5B
STAR
KCNK3
SCD
CLCF1
RHOBTB1
JUNB
CDC42EP4
PTPRJ
NAV2
SQSTM1
BIRC3
TRAF1
CSF2
RRAD
DMSO/SM (++)
CXCL1
MAP3K14
CXCL3
CXCL8
NFKBIA
NFKBIE
NFKB2
TNFAIP3
NUAK2
JUNB
ZNF697
KGN Cells
DMSO/R-RA
LIN28B-AS1
ADAT3
MARVELD2
DMSO/S-R-RA
INSIG1
MVK
HMGCR
RBM14
TFAP4
MAP3K14
NFKB2
NFKBIA
DMSO/SM
JUNB
COV434 Cells

Aim 3. To determine the mechanistic consequences of XIAP inhibition and PPARγ activation at the proteomic level in GCT.

Rationale

XIAP is an exciting target for cancer therapeutics (Fulda 2015). As XIAP (compared to other IAP members) is predominantly expressed in GCT, there is a strong rationale for elucidating the role of XIAP using the GCT-derived cell lines. XIAP acts primarily via protein–protein interactions and E3 ubiquitin ligase activity. Identification of proteins that either directly or indirectly associate with XIAP may lead to further discovery of therapeutic targets for the treatment of other cancers, not just GCT. This is supported by the development of Smac mimetics designed according to the structural features of the XIAP–Smac interaction (Fulda and Vucic 2012).

Aim 3.1: To determine the XIAP functional domains involved in PPARγ-mediated apoptosis

We have used extensively characterized lentiviral XIAP expression constructs that have been re-introduced into XIAP-deficient GCT-derived cell lines. The aim is to compare XIAP proteins with mutations in the functional domains in order to determine in order to determine the role of the functional domains involved in PPARg-mediated apoptosis. We are utilizing stable isotope labeling with amino acids in cell culture (SILAC) combined with mass spectrometry to identify proteins involved in the response.

As a proof-of-concept, we initially performed SILAC using KGN cells grown in vehicle-treated media supplemented with light amino acids (lysine and arginine) as the control, and performed a proteomic comparison using cells grown in medium supplemented with heavy amino acids (lysine and arginine) and the combination treatment.

Altogether, a total of 569 proteins were identified in the KGN cells after CmpdA/RGZ/RA or Emb/RGZ/RA treatment. Of these, 22 were upregulated and 10 downregulated by ≥1.5 fold after the combined treatment (**Table 1 and 2**).

Table 1. Upregulated proteins in KGN cells.[1]

Protein ID	Gene	Protein	CmpdA/RGZ/RA Average ratio	Emb/RGZ/RA Average ratio	Peptides identified	Protein score	% Sequence coverage
O00767	SCD	Stearoyl-CoA desaturase	4.50	4.59	5	87 344	3.6 10.6
P08648*	ITA5	Integrin alpha-5	nd	3.23	3	- 53	2.6 4.1
P00558	PGK1	Phosphoglycerate kinase I	nd	2.87	18	875 1118	55.6 53.5
O95573	ACSL3	Acyl-CoA synthetase long chain family member 3	nd	2.56	6	51 84	4.4 5.6
P07602	PSAP	Prosaposin	1.79	2.32	10	CmpdA/RGZ/RA: 361 190 Emb/RGZ/RA: 177 240	CmpdA/RGZ/RA: 22.1 9.4 Emb/RGZ/RA: 7.3 19.3
P21980*	TGM2	Protein-glutamine gamma-glutamyltransferase 2	2.30	nd	-	- 221	2.6 9.3
P08670	VIM	Vimentin	2.21	nd	40	4165 2682	84.5 69.5
P07858	CTSB	Cathepsin B	1.56	1.97	7	CmpdA/RGZ/RA: 225 254 Emb/RGZ/RA: 170 362	CmpdA/RGZ/RA: 19.2 18 Emb/RGZ/RA: 10 23.9
P61769	B2M	Beta 2 microglobulin	1.74	1.90	2	CmpdA/RGZ/RA: 83 28 Emb/RGZ/RA: 79 141	CmpdA/RGZ/RA: 26.9 26.9 Emb/RGZ/RA: 26.9 26.9
P04083	ANXA1	Annexin A1	nd	1.83	20	759 43	50.9 2.7
P11279*	LAMP1	Lysosome-associated membrane glycoprotein 1	nd	1.75	3	- 134	6.8 14.4
Q16777	H2A2C	Histone H2A type 2-C	1.70	nd	5	273 95	35.9 14
P00338	LDHA	L-lactate dehydrogenase A chain	nd	1.69	21	158	15.7
P13473*	LAMP2	Lysosome-associated membrane glycoprotein 2	nd	1.67	3	- 1422	9.7 72.9
Q13162	PRDX4	Peroxiredoxin-4	nd	1.67	6	84 290	16.6 21
P60174	TPI1	Triosephosphate isomerase	nd	1.64	12	693 705	54.2 52.8
P07237	P4HB	Protein disulfide isomerase	nd	1.59	9	149 274	12.8 15.9
P45880*	VDAC2	Voltage-dependent anion-selective channel protein 2	1.57	nd	-	- -	5.7 9.9
P17516*	AKRIC4	Aldo-keto reductase family 1 member C4	1.56	nd	-	133 58	15.8 8
P06733	ENO1	Alpha enolase	nd	1.55	31	1456 1790	68.4 78.6
P11021	HSPA5	78 kDa glucose-regulated protein	nd	1.52	21	828 1299	29.5 37
P07339	CTSD	Cathepsin D	1.51	nd	17	606 1284	26.9 46.4

Table 2. Downregulated proteins in KGN cells.[2]

Protein ID	Gene	Protein	CmpdA/RGZ/RA Average ratio	Emb/RGZ/RA Average ratio	Peptides identified	Protein score	% Sequence coverage
P49411	TUFM	Elongation factor Tu, mitochondrial	nd	-2.7	13	14 719	14 30.5
O94925*	GLSK	Glutaminase	-1.64	nd	1	234 234	5.4 5.4
Q16695	HIST3H3	Histone H3.1	nd	-1.99	8	289 300	54.4 54.4
Q07021*	C1QBP	Complement component 1Q subcomponent-binding protein, mitochondrial	nd	-1.98	-	- -	14.5 14.5
P35579	MYH9	Myosin-9	nd	-1.81	32	1952 3291	30.1 36.6
P40926*	MDHM	Malate dehydrogenase	nd	-1.76	-	- 54	13.4 21.6
P05141	SLC25A 5	ADP/ATP translocase 2	nd	-1.57	9	174 195	29.2 14.8
P62249*	RS16	40S ribosomal protein S16	nd	-1.51	1	- 75	23 23
P62263*	RS14	40S ribosomal protein S14	nd	-1.50	-	- -	10.8 10.8
Q16658	FSCN1	Fascin	-1.65	nd	4	277 188	8.5 7.5

We then used the *Search Tool for the Retrieval of Interacting Genes/Proteins* (STRING v10) to identify any interactions between the regulated proteins and classify these proteins according to their biological processes. GO enrichment analysis identified several subsets of proteins which were enriched for various steps involved in the canonical glycolysis pathways (P<0.05) (Table 3). The significant upregulation of metabolic pathways is consistent with the restoration of PPARγ activity upon removal of the NF-κB transrepression by the XIAP inhibitors, CmpdA and Emb. No pathway was found to be over- or under-represented amongst the downregulated proteins.

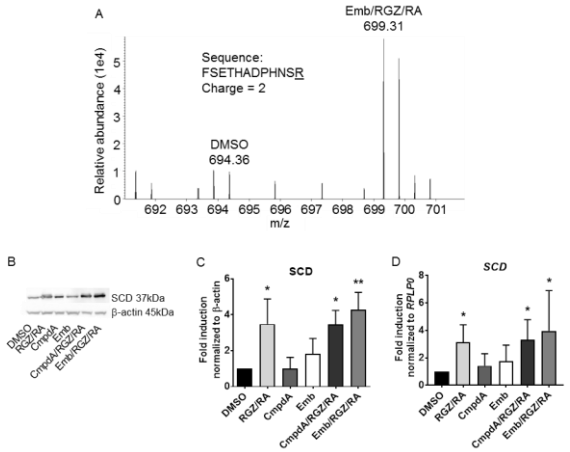
Table 3. GO enrichment analysis.[3]

GO biological process	Homo sapiens REF #	#	Expected value	Fold enrichment	*P* value
Canonical glycolysis	27	3	0.02	>100	0.01
- Glucose catabolic process to pyruvate	27	3	0.02	>100	0.01
- Pyruvate metabolic process	69	4	0.05	75.99	0.00175
- Monocarboxylic acid metabolic process	481	6	0.37	16.35	0.00812
- Carboxylic acid metabolic process	868	8	0.66	12.08	0.000698
- Oxoacid metabolic process	976	8	0.74	10.74	0.00172
- Organic acid metabolic process	993	8	0.76	10.56	0.00196
- Single-organism cellular process	9841	16	7.51	2.13	0.0469
- Single-organism metabolic process	3572	12	2.73	4.40	0.00465
	30	3	0.02	>100	0.0137
- Glucose catabolic process	108	4	0.08	48.55	0.0103
- Single-organism carbohydrate catabolic process	115	4	0.09	45.59	0.0133
	847	7	0.65	10.83	0.0123

Among the 22 upregulated and 10 downregulated proteins, stearoyl-CoA desaturase (SCD) showed the greatest fold of change after CmpdA/RGZ/RA treatment compared to vehicle. Orbitrap mass spectrometry analysis identified 4.5- and 4.6-fold (average change of reciprocal experiments) of induction in SCD in the KGN cells after combined CmpdA/RGZ/RA and Emb/RGZ/RA treatment, respectively (**Fig. 2A and Table 1**).

In KGN cells, western blot analysis demonstrated that SCD protein levels were significantly increased after CmpdA/RGZ/RA (3.5 fold) and Emb/RGZ/RA (4.3 fold) treatment (**Fig. 2B and C**) confirming the mass spectrometry findings. We also investigated the response of SCD at the messenger RNA level. SCD is encoded by the stearoyl-CoA desaturase (*SCD*) gene. Digital PCR using the Fluidigm® Biomark HD™ system demonstrated that *SCD* mRNA was upregulated by 3.3- and 4.0-fold after 24-hour treatment with CmpdA/RGZ/RA and Emb/RGZ/RA in the KGN cells, respectively (**Fig. 2D**).

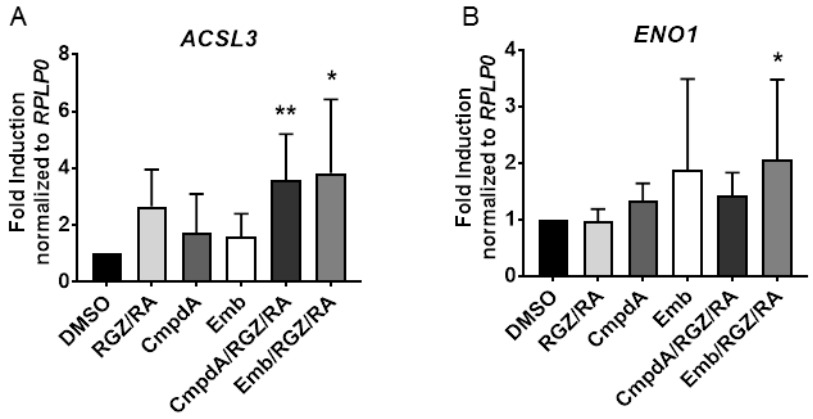
Figure 2



Upregulation of proteins and genes associated with metabolism

Besides SCD, functional annotation of differentially regulated proteins performed by GO enrichment analysis revealed an over-representation of the canonical glycolysis pathway ($P<0.05$) (**Table 3**). Following selection based on the magnitude of change, we quantified the mRNA levels of the mRNA that encode for the proteins involved in this biological process using the Fluidigm Biomark HD system. In addition to SCD, we also identified other targets that are regulated by the combined PPARγ and XIAP treatment. These include acyl-CoA synthetase long-chain family member 3 (ACSL3) and α-enolase (ENO1). ACSL3 protein levels were significantly increased by Emb/RGZ/RA (**Table 1**) and *ACSL3* gene expression was increased by both CmpdA/RGZ/RA and Emb/RGZ/RA treatment (**Fig. 3A**). Similarly, ENO1 demonstrated a consistent induction at both the mRNA (**Fig. 3B**) and protein (**Table 1**) levels following Emb/RGZ/RA treatment.

Figure 3

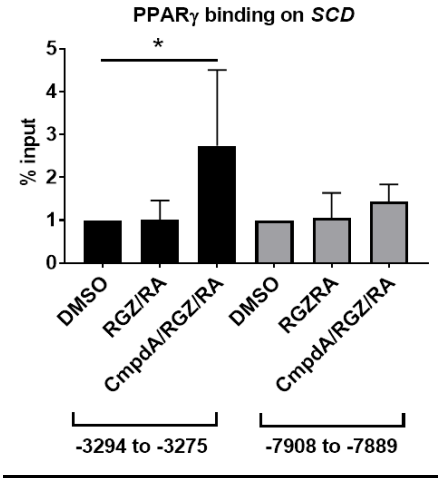


PPARγ binding sites on SCD promoter region

Our screening identified two putative binding sites for PPARγ between nucleotides point -7908 to -7889 and -3293 to -3274 relative to the start site of transcription.

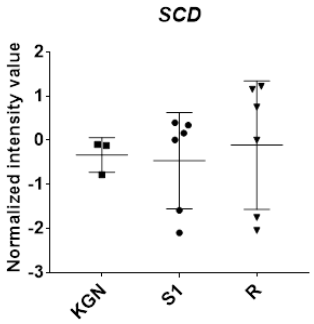
Following 6-hour of drug treatment with DMSO, RGZ/RA or CmpdA/RGZ/RA, ChIP-PCR analysis demonstrated a significant increase in PPARγ binding at the region -3293 to -3274 (**Fig. 4**). There appears to be a small increase at -7908 to -7889, however, this is not significant (**Fig. 4**).

Figure 4.



Detection of SCD, ACSL3 and ENO1 in untreated KGN cells and human GCT samples
We have previously performed a whole transcriptome analysis of 12 human GCT and the KGN cells which enabled us to analyze the levels of *SCD, ACSL3* and *ENO1* mRNA in untreated KGN cells and human GCT using this data set. *SCD* mRNA was detected in all samples. The levels do not differ by stage. The stage (stage 1 versus recurrent) of the disease does not alter the level of expression; the KGN cells showed similar level of *SCD* mRNA as the human GCT samples (**Fig. 5**). Similarly, ACSL3 and ENO1 were both detected in all samples with similar levels of expression in the stage 1 and recurrent GCT (Data not shown). Statistical analysis was not performed with only one cell line from 3 replicate experiments included in the data set.

Figure 5.



Works in Progress:
We are now preparing XIAP mutant expressing GCT-derived cell lines for SILAC treatment, and perform proteomic analysis as shown above and described in **Aim 3.2 and 3.3** in the Research Narrative. COVID-19 has prevented access to the Mass Spectrometry facility to do this analysis, however, we anticipate being able to submit our samples in December of 2020.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

We have disseminated findings of our research and publications to:
Granulosa Cell Tumor Research Foundation
Ovarian Cancer Research Foundation
Monash Health Department of Gynaecological Oncology

Newsletters:
http://www.monashpartnersccc.org/wp-content/uploads/2019/09/MPCCC-Annual-Report-2018-2019_interactive_2.pdf (Page 27)

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We are in the middle of completing some very important experiments as described in the Statement of Work. Due to COVID19 – our state has been in lockdown since April 2020 (7 months), which has severely impacted on progress of a number of these experiments. After commencement of our research activities from Oct 2020, we are slowly going back to normal, and will be very active over the next 12 months to complete the rest of our experiments.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our findings to show a combination approach using a cancer drug that reduces the granulosa cell tumor's defense mechanisms together with an anti-diabetic drug that attacks the cancer cells. These two drugs are already in clinical or pre-clinical use for other conditions making it likely that we can move quickly from a testing phase into the treatment of this form of ovarian cancer.

The major finding so far is that this combination approach has been effective not only with cell grown in culture flasks, but particularly for cells grown in 3D – which more closely resembles that actual tumor microenvironment. In addition, we have tested the efficacy on surgical tissue samples from GCT patients, that are grown in the dish, and found that the combination approach is just as effective. This has led us to planning in performing these studies in animal models.
Furthermore, we have identified some important genes that are regulated as a consequence of the combination approach, of which may have implications for resistance to the treatment, and provide potential clues as to effectiveness of the treatment, and alternatives in the case of resistance.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Impact of COVID-19

Our state (Victoria, Australia), has experienced an extensive lockdown period due to 2 waves of the COVID-19 pandemic. The restrictions imposed on our workforce are:
From 21st March 2020, only essential services were allowed to operate, with a stay-at-home directive given to all staff. This severely curtailed staff in performing labwork, and we made provision to use the lockdown period to analyze data online. We are still under these restrictions, including a curfew, as well as a 5-kilometer limit that one can travel from their residence (enforced until Oct 2020). A gradual lifting of these restrictions is in progress, and staff are allowed back on a roster basis. We anticipate a return to normal working output in December 2020.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Aim 1.1 Developing an in vivo xenograft model

The *in vivo* work outlined in the grant has been delayed due to a number of reasons. The primary delay has been the animal facility located at our Institute has encountered issues centering on construction works of a nearby building that has affected the breeding patterns of animals. The difficulties of performing our work at the Hudson Animal Facility has been a source of frustration, and have caused a delay in my seeking ethics until I have been sure that I can perform the experiments in a suitable facility with minimal likelihood in interruptions to the experiments. Hence, I have only just begun to initiate animal ethics applications that will now utilise the animal facilities at nearby Monash University in collaboration with a researcher at the Biomedical Discovery Institute (BDI) at Monash University. A benefit of this is that we can incorporate the findings from Aim 2.1 and Aim 2.2 into the ethics application, and the animal studies can be carried out concurrently with this aim.

Additionally, the original animal imaging technology proposed was decommissioned due to failure of the laser source. To resolve this issue, the facility located at BDI, Monash University has a newly acquired AMI HTX Small Animal Optical Imager which brings better capabilities to our proposed imaging needs for the *in vivo* component of the project.

As described above, COVID-19 has also become an interruption to our workflow, as currently our State has experienced considerable lockdown periods that have disrupted lab operations since April 2020. We are hoping that our laboratory work will be allowed to restart within the next few weeks. This has also had implications regarding applications for animal ethics, as the committees are meeting irregularly, and hence significant delays in assessing and finalising applications.

I anticipate that we will be able to receive our animal ethics approvals by Dec 14th 2020, and will be able to commence our animal studies by February 2021.

Aim 2.1: To investigate PPARγ DNA interactions at a genome-wide level

Works in Progress:
We have processed all samples in order to perform ChIP-Seq. The samples were ready to go to the next stage of library preparation, however, as a result of our state's COVID-19 lockdown, there has been a delay in getting these samples to the genomics facility for generation of the libraries and subsequent sequencing. We anticipate submission of these samples now from the week of 12h October 2020.

Aim 3.2 and 3.3: To identify the proteins involved in XIAP post translational modification and to validate and characterize regulated proteins

Works in Progress:
We are now preparing XIAP mutant expressing GCT-derived cell lines for SILAC treatment, and perform proteomic analysis as shown above and described in **Aim 3.2 and 3.3** in the Research Narrative. COVID-19 has prevented access to the Mass Spectrometry facility to do this analysis, however, we anticipate being able to submit our samples in December of 2020.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

We have 2 papers in preparation for submission in Jan/Feb 2021

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nil

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Presentations:
Seminar Presentation to Victorian Comprehensive Cancer Centre 13th Aug 2020 "Granulosa Cell Tumors: how are they different and why do they matter?" (https://www.youtube.com/watch?v=J_U4TN4iOlQ)

Rare Ovarian Cancer Incorporated (ROCInc) Webinar 1 March 2020: "Granulosa Cell Tumour GCT, a rare ovarian cancer Q&A on Treatment, Challenges and Research" (https://www.youtube.com/watch?v=EcZwsmXwCo0)

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

http://www.monashpartnersccc.org/wp-content/uploads/2019/09/MPCCC-Annual-Report-2018-2019_interactive_2.pdf (Page 27)

Monash Partners Comprehensive Cancer Consortium (MPCCC) is a strategic alliance of health service and research organizations working in partnership to improve outcomes for people affected by cancer. Our work was featured as part of their annual report for research activities performed in 2018 – 2019.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

  Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Trang Nguyen
Project Role:	Research Assistant
Months worked:	27 months
Contribution to Project:	Ms Nguyen performed RNA-Seq, ChIP, and XIAP knockout experiments, performs all Tissue Culture experiments. She will be involved in the in vivo experiments beginning 2021.
Name:	Maria Alexiadis
Project Role:	Senior Research Assistant (0.5 FTE)
Months worked:	12 months
Contribution to Project:	Ms Alexiadis is performing Bioinformatic analysis for our RNA-Seq data, proteomic analysis of SILAC experiments, and will be analyzing the ChIP-Seq data once performed in conjunction with Professor George Muscat.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*