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TITLE: Novel Tumor Suppressor Gene in Hereditary X-linked Ovarian Cancers

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CONTRACTING ORGANIZATION: Health Research, Inc.

Buffalo, New York 14263-0001

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14. ABSTRACT

Our previous work has identified an ovarian cancer risk locus on the X-chromosome and likely within the gene MAGEC3. The overall goal of this proposal is forward the idea that MAGEC3 is a tumor suppressor gene and to determine the scope and impact of the mechanism. Aim 1 is focused on studying the mechanism of MAGEC3 silencing in clinical samples. Aim 2 uses cell lines to study the function of MAGEC3 and Aim 3 will confirm our findings in mouse xenograft models. We determined that candidate tumor suppressor MAGEC3 is highly likely to be transiently expressed and cell cycle regulated with tight epigenetically-related expression of the protein increasing the confidence that it is a tumor suppressor gene. This result was achieved through the engineering of multiple cell lines with inducible MAGEC3 expression and transgene tags that will enable continuing RNA and protein level analyses. Single cell level expression analyses confirmed cell cycle association and flow cytometry protein-level analysis also supports these findings.

15. SUBJECT TERMS

Cancer antigen, cancer genetics, DNA repair, genetic epidemiology, ovarian cancer, tumor suppressor gene.

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1) Introduction

Our previous work has identified an ovarian cancer risk locus on the X-chromosome and likely within the gene MAGEC3. The overall goal of this proposal is forward the idea that MAGEC3 is a tumor suppressor gene and to determine the scope and impact of the mechanism. Aim 1 is focused on studying the mechanism of MAGEC3 silencing in clinical samples. Aim 2 uses cell lines to study the function of MAGEC3 and Aim 3 will confirm our findings in mouse xenograft models. We determined that candidate tumor suppressor MAGEC3 is highly likely to be transiently expressed and cell cycle regulated with tight epigenetically-related expression of the protein increasing the confidence that it is a tumor suppressor gene. This result was achieved through the engineering of multiple cell lines with inducible MAGEC3 expression and transgene tags that will enable continuing RNA and protein level analyses. Single cell level expression analyses confirmed cell cycle association and flow cytometry protein-level analysis also supports these findings.

2) Keywords

Cancer antigen, cancer genetics, DNA repair, genetic epidemiology, ovarian cancer, tumor suppressor gene.

3) Accomplishments

What were the major goals of the project?

Specific Aim 1	Timeline	Progress
Major Task 1 is to analyze selected familial cases from FOCR and RPCI Biobanks.		
Local IRB Approval: CIC95-27.	0	Complete
Sub Task 1. Conduct germline/somatic WES, X chromosome sequencing, RNA sequencing, Methylation.	1-8	Complete
Sub Task 2. Conduct genetic analyses.	8-12	Complete
Major Task 2 is to analyze sporadic cases from the RPCI biobank.		
Sub Task 1. Receive and qualify WGS data from APOLLO collaborators.	1-3	Complete
Sub Task 2. Conduct germline/somatic WES, X chromosome sequencing, RNA sequencing, Methylation.	1-6	Complete
Sub Task 3. Conduct genetic analyses.	6-10	Complete
Sub Task 4. Correlative studies with clinical and pathological variables and outcomes.	8-12	Partial- in progress
Specific Aim 2		
Major Task 3 is to characterize ovarian cancer cell line phenotypes in response to MAGEC3		
Sub Task 1. Construct MAGEC3 shRNA knockdown lines	13-18	Partial – in progress
Sub Task 2. Construct lentiviral MAGEC3 lines	13-18	Completed
Sub Task 3. Characterize cell lines'	18-30	Partial – in

proliferative phenotype and MAGEC3 expression. Perform statistical analyses.		progress
Specific Aim 3		
Major Task 4 is to assess the tumorigenic potential of modified MAGEC3 cell lines		
Sub Task 1. Obtain IACUC Approval	18-21	Complete
Sub Task 2. Pilot intrabursal study.	21-24	Delayed
Sub Task 3. Perform xenograft studies, isolate and collect tumors. Conduct statistical analyses.	24-36	Not Started
Major Task 5 is to prepare manuscripts for submission		Partial – in progress

What was accomplished under these goals?

Accomplished under major task 1.

In this reporting period, our IRB/HRPO plan was approved.

Familial history samples with whole genome sequencing were evaluated for MAGEC3 variants, however none were observed. For reference, BRCA1 and BRCA2 mutations in these samples were provided. Recall that these samples were selected for reported "BRCA-negative" familial status so it was not unexpected to find a few variants.

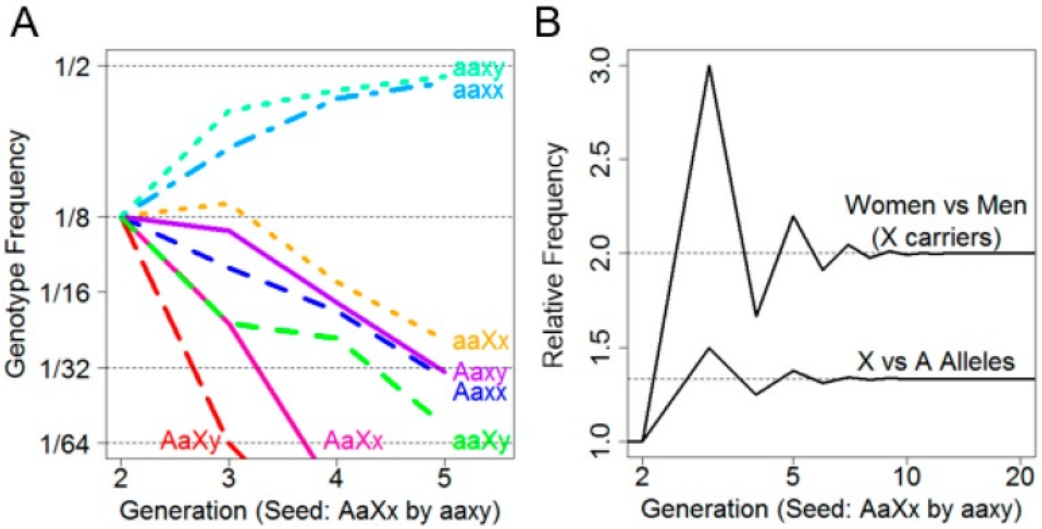
Table 1.
FOCR Samples (N=50 genomes)

	BRCA1	BRCA2	MAGEC3
Frameshift Deletion	2	0	0
Frameshift Insertion	3	1	0
Non-synonymous SNV	64	29	0
Synonymous SNV	25	0	0

We conducted pedigree analyses of the selected families in the FOCR combining new and pre-existing sequencing data. Using this information we developed a transmission model and made mathematical predictions about the rate of carriers. These models are complicated by the confounding of the risk allele and the individual's sex. We were able to devise a screening strategy for the better identification of X-linked families. This model, information and strategy is intended to be disseminated to a lay audience for easier identification of the X-linked hereditary ovarian cancer pattern.

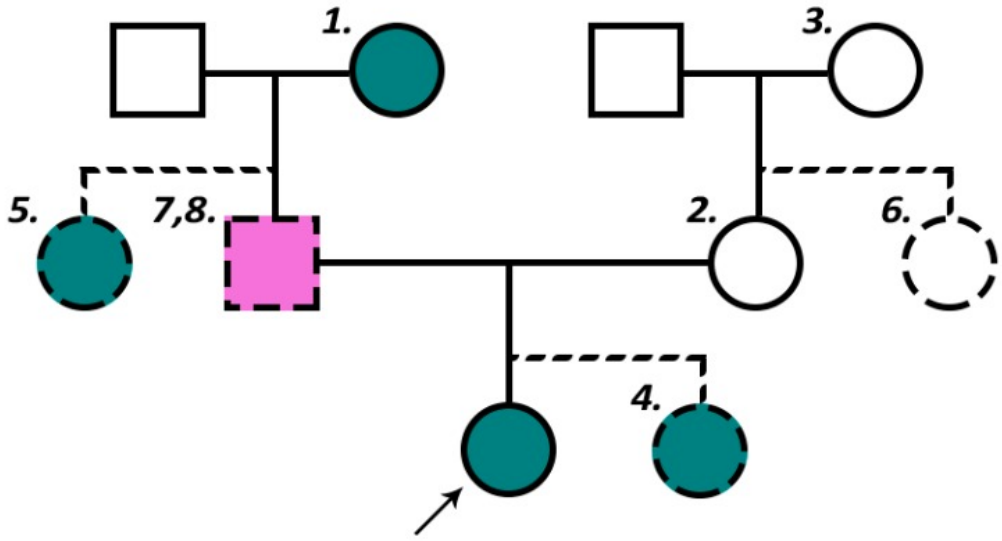
Assuming mates from outside the family carry no risk alleles, a key observation of the modeling is that female X-linked carriers are more likely to persist in descendants than autosomal carriers. However, male carriers more rapidly convert to non-risk alleles. The effect is compounded when considering multiple offspring.

Figure 1. Mathematical modeling of rates of transmission of decay at an autosomal risk locus {A,a} versus an X-linked locus {X,x,y}. X-linked risk alleles persist longer than other alleles (A) and are more likely to appear in women in the long run (B).



Based on the three-generation pedigree and the review of pedigrees in the FOCR cohort, we devised the following scheme for screening and published it in Diagnostics (Etter et al., 2020, PMID 32046210).

Figure 2. Template pedigree for screening recommendations. Key individuals in the pedigree are numbered, ovarian cancers are colored teal, putative prostate cancer is colored pink, and the proband is indicated by an arrow.



Accomplished under major task 2

In this reporting period, our IRB/HRPO plan was approved.

As in major task 1, sequencing of sporadic cases also failed to identify MAGEC3 carrying cases.

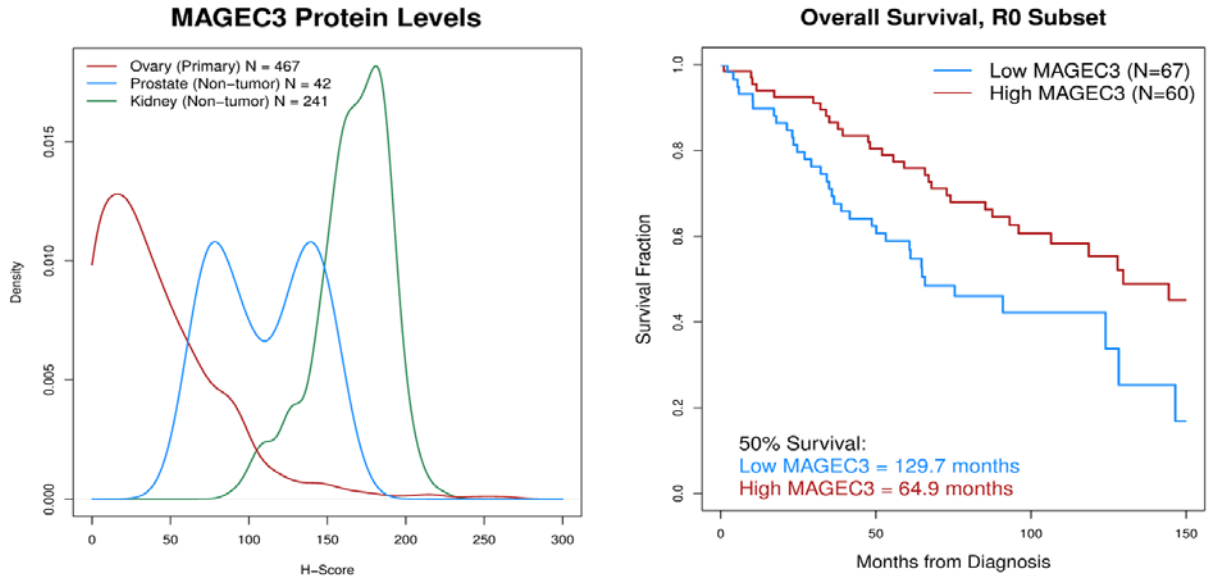
Table 2.
RPCI Cohort Samples (N=28)

	BRCA1	BRCA2	MAGEC3
Frameshift Deletion	0	0	0
Frameshift Insertion	1	0	0
Non-synonymous SNV	0	1	0
Synonymous SNV	0	0	0

Having observed few mutations, we proceeded with our plan to investigate the level of MAGEC3 regulation (DNA/protein/RNA) and investigated protein expression of MAGEC3. We used quantitative immunohistochemistry with an image processing algorithm in ImageJ to quantify the prevalence and intensity of staining.

We compared high stage primary ovarian cancer expression in n=467 cases stained on an institutional tissue microarray. Because non-tumor controls were not available for these high stage cases, we compared to a set of non-tumor prostate (male only) and kidney (male and female) controls to observe that there was indeed expression of MAGE at the protein level. MAGEC3 levels in the ovarian cases were split at the all-comers (ovarian primary) median which corresponded to the lowest observed non-tumor level. Among women achieving R0 after maximal debulking surgery, MAGEC3 was a significant predictor of poor survival split at the median (log rank p<0.01). Additional work is required to model the survival in the context of other clinical prognostic markers is required.

Figure 3. Protein MAGEC3 levels (measured by IHC H-score) for ovarian primaries, and non tumor from prostate and kidney cancer cases. Overall survival stratified by MAGEC3 expression (overall median).



Clinical correlates were available for the patients assayed. While MAGEC3 was predominantly downregulated at the protein level, higher MAGEC3 expression was associated with stage IIIC cancers specifically and lower progression free survival.

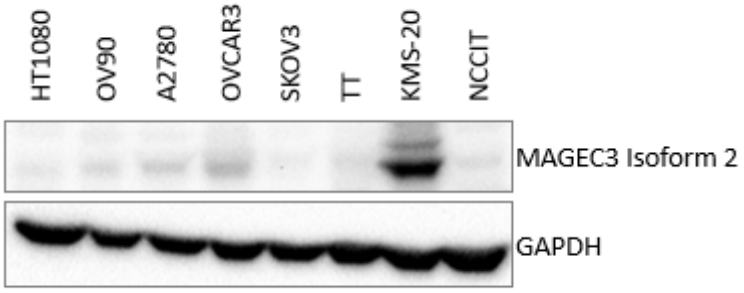
Table 3. Clinical correlates of protein MAGEC3 expression

		MAGEC3 H-score (Median Split)		
		High	Low	P-value
	n	234	233	
Age at Diagnosis	Age of Dx	64	62.2	0.20
FIGO Stage	I/II/IIIA/B	35	52	0.02
	IIIC	166	135	
	IV	29	38	
Grade	Well	55	62	0.60
	Moderate	152	139	
	Poor	25	25	
Histology	Serous	174	158	0.10
	Other Epithelial	60	75	
Cytoreduction	R0	60	67	0.40
	Not R0	172	159	
Overall Survival	Months	39.8	46.5	0.07
Progression-Free survival	Months	16.9	24.6	9.00E-04

Accomplished under major task 3

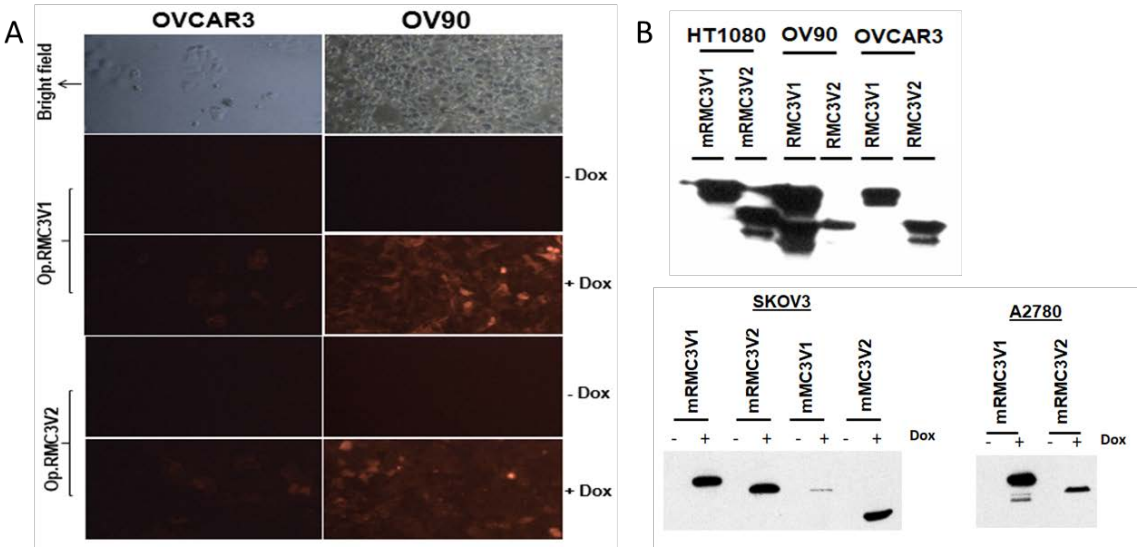
We have had mixed results continuing to look for endogenous MAGEC3 in ovary cancer cell lines. One approach was to consider the high expressing lines reported in the Cancer Cell Line Encyclopedia (TT, KMS-20) as well as a testicular cancer line (NCCIT) given the expectation that most MAGE genes are cancer testis antigens.

Figure 4. MAGEC3 isoform 2 protein expression in ovarian cancer cell lines and negative control (HT1080) as well as lines with mRNA expression per CCLE (TT, KMS-20) and one testicular line (NCCIT).



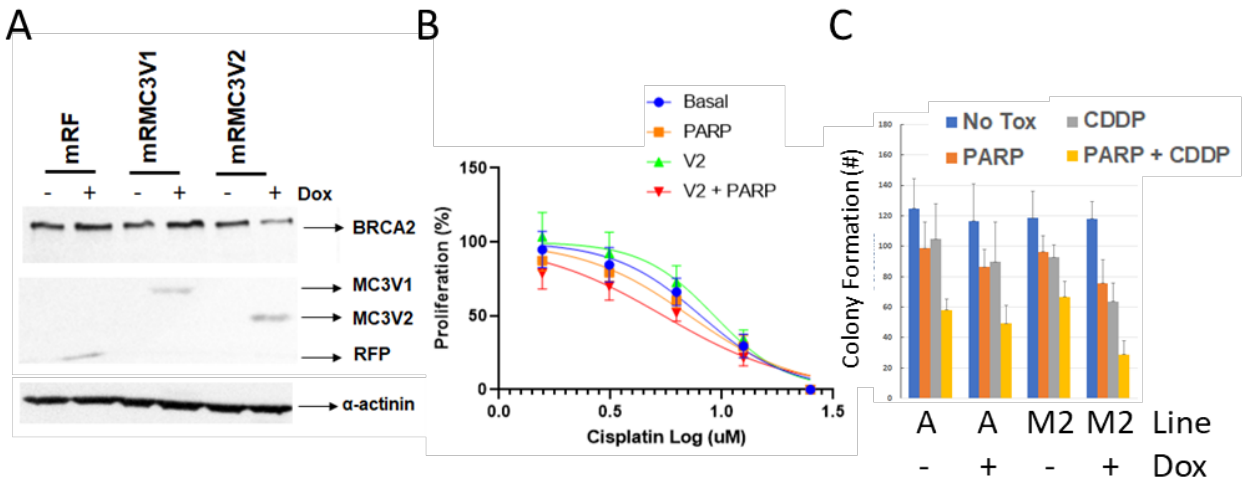
We reported in the previous period that few cell lines were found that expressed endogenous MAGEC3. To address this deficit, we have now generated SK-OV-3, A2780, OV90 and OVCAR3 ovarian cancer lines with inducible MAGEC3 on a tetracycline promoter. This heterologous MAGE is myc-tagged and RFP-tagged for easy identification. Lines without the RFP tag have also been produced.

Figure 5. Stable transfection of both isoforms of MAGEC3 with established with an RFP tag (suffix: Op.mRMC3V1, Op.mRMC3V2). Example of observed inducible expression of MAGEC3 isoforms in OVCAR3 and OV90 with RFP tag (A). The western blot analysis to confirm the MAGEC3 induction of the established cell lines (B). The SKOV3 cell lines had separate lines made without the RFP tag.



Pilot experiments in HT1080 (sarcoma line) suggest that MAGEC3 regulates BRCA2 and this combination modulates response to platinum and PARP inhibition. These experiments will be recapitulated in the newly derived ovarian cancer lines and may motivate the study of further augmenting PARP therapy.

Figure 6. MAGEC3 isoform 2 leads to PARP/CDDP synergy through reduction of BRCA2 protein. Western blot of induced MAGEC3 isoforms in HT1080 (sarcoma) indicate a reduction in BRCA2 protein (A). MTS assay indicates that induced MAGEC3 isoform 2 (V2) synergizes with PARP inhibition (fixed dose) to augment response to platinum (B). Replication of synergy phenotype using colony formation assay line A is the RFP transfected control, M2 is tetracycline inducible isoform 2 (C).



Accomplished under major task 4

We obtained ACURO approval on schedule for animal experiments in year 3. The remainder of task 4 is scheduled for year 3 of this award.

Accomplishments under major task 5

Submission and acceptance of

- Tsuji T*, Eng KH*, Matsuzaki J, Battaglia S, Szender JB, Miliotto A, Bshara W, Morrison C, Lele S, Emerson R, Wang J, Liu S, Robins H, Lugade A, Odunsi K. (2020) Clonality and antigen-specific responses shape the prognostic effects of tumor-infiltrating T cells in ovarian cancer. Oncotarget. Accepted. *Co-first author

Submission and publication of

- Etter JL, Moysich K, Kohli S, Lele S, Odunsi K, Eng KH. Transmission of X-linked Ovarian Cancer: Characterization and Implications. Diagnostics (Basel). 2020;10(2):90. PMID: 32046210

Other achievements
Nothing to report.

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

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

What do you plan to do during the next reporting period to accomplish the goals?

We will continue our bench experiments exploring the regulation of MAGEC3 at the protein level.

We will begin in vivo experiments testing the modulation of response to platinum and/or PARP by MAGEC3 expression.

We have a number of manuscripts in preparation for the next reporting period.

Manuscript 1 – MAGE phenotyping

- Results showing that MAGEC3 is cell cycle regulated
- Results showing the mRNA response to platinum adaption under MAGEC3 expression

Manuscript 2 – Clinical MAGE

- Clinical correlates of MAGEC3 protein expression.

Manuscript 3 – MAGE/BRCA2 regulation.

4) Impact

What was the impact on the development of the principal disciplines of the project?
We have determined that MAGEC3 silencing does not occur at the DNA level. This has refocused our studies on the regulation of protein MAGEC3.

We have preliminary evidence that MAGEC3 modulates DNA repair capabilities in multiple cell lines which introduces the potential for augmenting PARP inhibition therapy.

What was the impact on other disciplines?
Nothing to report

What was the impact on technology transfer?
Nothing to report

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

5) Changes/Problems

Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Due to COVID19, our institution shut down research operations in March 2020 interrupting the planned pilot *in vivo* studies and terminating a number of cell culture projects. We are permitted to maintain a limited staff on site: in June 2020, we are at presently 50% operational staffing.

Our institution is planning to reopen formally under the New York State phased reopening plan and will be fully functional after Phase IV. At that time, we will assess the state of our planned grant activities. Access to our shared resource facilities has been limited during this time and we anticipate a backlog of work.

Changes that had a significant impact on expenditures
We had significant delays in hiring research staff. Under our institutional COVID19 policy, we were forced to discontinue the use of the research apprentice title and to implement a reduction in work force.

Significant changes in use or care of human subjects, vertebrate animals, biohazards and or select agents

Nothing to report.

6) Products

Journal publications

- Etter JL, Moysich K, Kohli S, Lele S, Odunsi K, Eng KH. Transmission of X-linked Ovarian Cancer: Characterization and Implications. Diagnostics (Basel). 2020;10(2):90. PMID: 32046210
- Tsuji T*, Eng KH*, Matsuzaki J, Battaglia S, Szender JB, Miliotto A, Bshara W, Morrison C, Lele S, Emerson R, Wang J, Liu S, Robins H, Lugade A, Odunsi K. (2020) Clonality and antigen-specific responses shape the prognostic effects of tumor-infiltrating T cells in ovarian cancer. Oncotarget. Accepted. *Co-first author

Websites or other internet sites
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications and or licenses
Nothing to report

7) Participants and other collaborating organizations

What individuals worked on the project?

Kevin Eng, PhD. PI, no change

Iqbal Aijaz, PhD. Postdoctoral Researcher. 8.4 Calendar Months.

- Dr. Aijaz is responsible for the design and execution of bench experiments and development of inducible cell lines.

Nicole Cavanaugh, BS. Research Apprentice. 4.8 Calendar Months.

- Ms. Cavanaugh conducts bench experiments under Dr. Aijaz's direction.

Sheethal Umesh Nagalakshmi, BS. Research Apprentice. 3.6 Calendar Months.

- Ms. Umesh Nagalakshmi conducted bioinformatic analyses and data QC during the project.

John Krolewski, MD PhD. Co-investigator. 0.7 Calendar Months.

- Dr. Krolewski assisted in the development and execution of the IACUC protocol and the animal models.

Has there been a change in the active other support of the PI or senior/key personnel since the last reporting period?

Previously pending awards were activated during this period.

	Level of support
DOD award W81XWH-19-1-0397 (PI: Eng)	15.0%
DOD award W81XWH-19-1-0378 (PI: Krolewksi)	8.0%
NIH award R01 CA247362-01A1 (PI: Knudsen)	5.0%
NIH award R01 CA247771 (PI: Lovell/Abrams)	1.9%

What other organizations were involved as partners?
Nothing to report

8) Special reporting requirements
Nothing to report

9) Appendices

None