AWARD NUMBER: W81XWH-18-1-0134

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

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REPORT DATE: JUNE 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| June 2019 | Annual | 15 May 2018 – 14 May 2019 |

**4. TITLE AND SUBTITLE**

Novel Tumor Suppressor Gene in Hereditary X-linked Ovarian Cancers

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-18-1-0134

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**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.

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| Unclassified | Unclassified | Unclassified | Unclassified | 11 | 19b. TELEPHONE NUMBER *(include area code)* |

**TABLE OF CONTENTS**


| | |
|---|---|
| Title Page | 1 |
| Standard Form (SF298) | 2 |
| Table of Contents | 3 |
| Introduction | 4 |
| Keywords | 4 |
| Accomplishments | 4 |
| Impact | 8 |
| Changes/Problems | 8 |
| Products | 8 |
| Participants and other collaborating organizations | 9 |
| Special reporting requirements | 11 |
| Appendices | 11 |

## 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.


## KEYWORDS

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


## ACCOMPLISHMENTS

**What were the major goals of the project?** The three aims are separated into five major tasks as outlined in the SOW.

- **Major Task 1 is to analyze selected familial cases from FOCR and RPCI Biobanks.**
- **Major Task 2 is to analyze sporadic cases from the RPCI biobank.**
- **Major Task 3 is to characterize cancer cell line phenotypes in response to MAGEC3**
- **Major Task 4 is to assess the tumorigenic potential of modified MAGEC3 cell lines**
- **Major Task 5 is to prepare manuscripts for submission**

**What was accomplished under these goals?**

**Accomplished under Major Task 1.**

We have received 58 cases of whole genome sequencing from the APOLLO/DOD group under our parent local protocol. We were unable to proceed with analysis outlined in the DoD award due to delays filing with HPRO. This delay originated from institution-level changes in coordinating sample and genomic data generation. As of this reporting period, our team has been briefed on the new workflow and filing local approval is underway. We expect to file with HPRO promptly upon receipt of local IRB determination.

In this reporting period, we have developed and tested our own pipelines (BWA > Picard > GATK > ANNOVAR) for germline WES/WGS that align, variant call and annotate for chromosome X and BRCA regions only using existing testing data from our institutional data banks. It currently takes about 1.1 days to complete a variant calling run.

**Accomplished under Major Task 2**. We have been delayed filing with HRPO due to the regulatory change referenced in major task 1. To mitigate this problem, we prepared separate internal protocols for Major Task 1 and Major Task 2 and have submitted them to our local IRB for determination. We expect to file with HPRO promptly upon receipt of local IRB determination and to update HPRO filings if approvals are received asynchronously.

**Accomplished under Major Task 3.**

We conducted RNAseq (**Table 1**), qPCR (**Fig 1**) and western blots to search for mRNA or protein MAGEC3 in several ovarian cell lines, but found no strongly expressing lines. This observation is not inconsistent with tumor suppressor function.

To address this limitation, we were able to express MAGEC3 via transient transfections into these lines (**Fig 2**), however the cells died quickly after transfection and we were only able to establish 2/8 stable clones. We attributed this difficulty to the tumor suppressive function of MAGEC3 and instead designed an inducible system that would introduce controllable MAGE levels after the cell recovers from infection. We developed stable lines in HT1080, PC3, A-498 lines and verified the

| Hg19, TopHat2, Gene Counts | IOSE-21 | HOSE-SV40 | SK-OV-3 | A2780 | OV90 | OVCAR3 |
|---|---|---|---|---|---|---|
| **MAGEC3** | 2 | 0 | 0 | 0 | 0 | 1 |
| **MAGEA3 (Control)** | 1789 | 1825 | 1490 | 1855 | 1888 | 2777 |
| **GAPDH (Control)** | 101595 | 75900 | 50120 | 48924 | 48908 | 68181 |

Table 1. RNA was isolated from normal and cancer ovarian cell lines and RNA sequencing was done in the Genomics Shared Resource. FASTQ files were aligned on the hg19 human genome version using TopHat2, and gene mRNA counts were quantified for MAGEC3.

controllable effect with qPCR (mRNA) and western blot (protein). These lines now exist for both isoforms of MAGEC3.

Preliminary experiments suggested that MAGEC3 induction augmented the response to hydrogen peroxide induced stress in HT1080 cells (p53 w/t, **Fig 3**) but not in PC3 cells (p53 null). This observation has led us to conjecture that MAGEC3, like other homologous MAGE-C family members, may directly bind or be regulated by p53. Again this proximity and interaction can be tested at the protein level by the BioID system and by simple co-immunoprecipitation. We will likely have to "add back" p53 to the PC3 cells to confirm the function.

The inserted MAGEC3 is Myc tagged and we have recently added an RFP tag for tracking; these tags will significantly improve the quality of protein work and the positive identification of the MAGEC3 protein. These lines also include variants with BirA, a promiscuous biotin ligase transgene from E. coli (the system is called BioID). When biotin is added to the cell culture, the BirA attached to MAGEC3 will tag neighboring proteins for identification. This will permit high-throughput protein-protein interaction screening and proximity screening (order ~10nm).

A fourth line 786-O was resistant to our selection vector. However, we managed to insert the MAGE plasmid and determined that the cells produce mRNA but silence the protein. We have determined that a myeloma line (MOLP-2) shows the same mRNA pattern with no protein. Inducible MAGEC3 was rapidly degraded in HT1080 cells after removal of doxycycline (**Fig 4**). Together these results imply that one way MAGEC3 is silenced is by the degradation of protein by a yet unidentified mechanism.

**Fig 1**. RNA was isolated from normal and cancer ovarian cell lines and MAGEC3 variant 1 and variant 2 mRNA levels were quantified by qRT-PCR. All samples were normalized to GAPDH mRNA levels and then compared to IOSE.

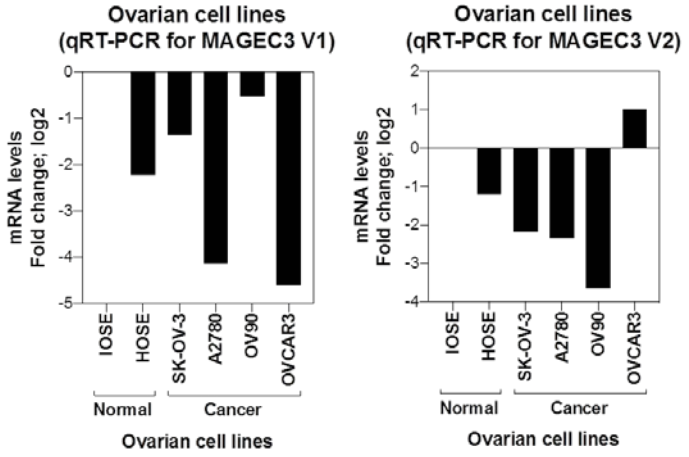
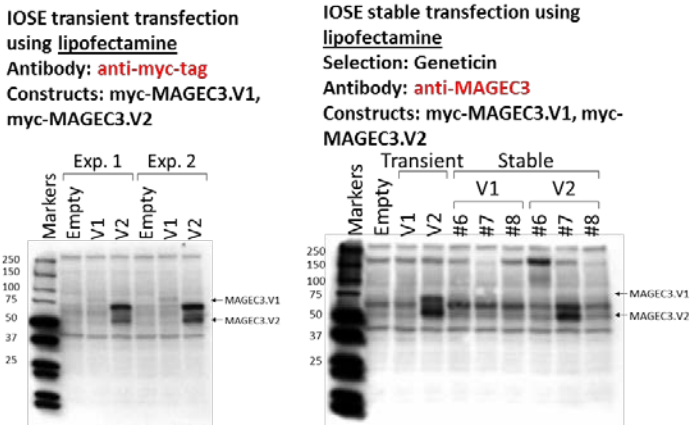



**Fig 2**. IOSE cells were transfected with myc-MAGEC3.variant1 or myc-MAGEC3.variant2 plasmids using lipofectamine transfection reagent. The experiment was done twice and proteins were isolated from all samples. (Left) Western blot was performed using an anti-myc-tag antibody to visualize the MAGEC3 protein levels. Cells transfected with an empty vector were used as reference. (Right) Stable transfections were executed and blotted using an anti-MAGEC3 antibody. Representative clones are shown here. Only clones 6 and 7 were stably transfected.




**Accomplished under Major Task 4.**

We have completed bulk RNA sequencing of ovarian (**Table 1**) and other cancer cell lines and will incorporate this data into future analyses.

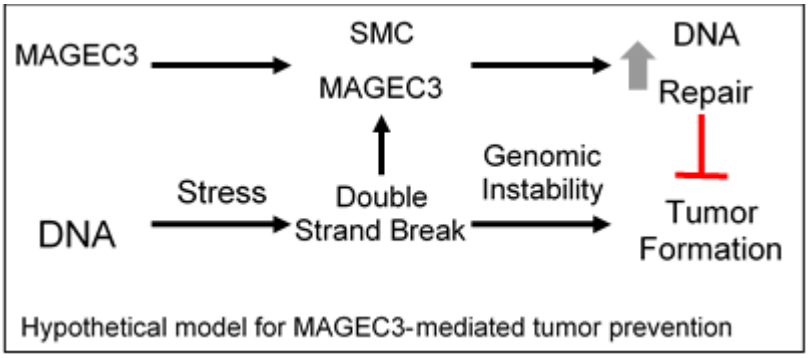
We had the opportunity to study MAGEC3 induction at the single-cell level using a new 10X Genomics Chromium system available at our institute (**Fig 5**). HT1080 cells infected with inducible MAGEC3 (isoform 2; our notation: MAGEC3.V2) were induced for 3 days and 30 days with a matching 3 day non-induced control. The lines were sent for single cell library preparation and sequencing yielding over 50,000 unique cells. We developed both an R/Seurat workflow and a R/SingleCellExperiment workflow to analyze this data.

In our findings, we learned that MAGE levels were often induced at a low and a high peak within reasonable biologic levels. MAGE induction was anti-correlated with the expression of many ribosomal protein mRNAs and positively

correlated with stress related genes (HSP90AA1, HSPE1); condensin/cohesin (SMC2/4, SMC3); and cell cycle genes (CKS1B, CKS2).

This observation led us to consider whether MAGEC3 was correlated with cell cycle (in the single cell mRNA data). Using the Seurat algorithms, we saw that cells expressing no MAGEC3 were mostly evenly distributed in cell cycle phase. Cells expressing increasingly higher levels of MAGE favored G2/M and not G1 in a dose-dependent manner (**Fig 6**). We recapitulated this finding using flow cytometry following the same experimental design: again we saw that protein MAGEC3 levels were associated with an increase in cells in G2. We confirmed that protein levels were constant at the 3 day and 30 day time points and validated the cell cycle association at the protein level using FACS (**Fig 7**). This finding strongly increased our confidence in the single-cell RNAseq findings and confirms that MAGEC3 is cell cycle regulated at the protein level.

These observations and our continuing literature review of other MAGE family members have led us to postulate the following testable model for the mechanism of MAGEC3 tumor suppressive effect: MAGEC3 should normally operate in a cell cycle dependent manner and associate with an SMC complex to effect DNA repair during G2 and prevent the formation of tumor. When MAGE's protective effects are disrupted, the cell is liable for tumor formation. The cell cycle association also explains why levels of MAGEC3 are hard to measure in bulk sequencing and highlights the need for a single-cell approach.




**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.

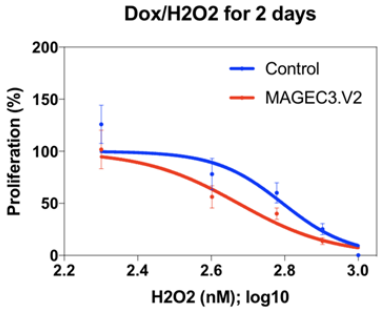



**Fig 3.** MAGEC3 expression by doxycycline (Dox) reduces proliferation in the presence of DNA damage ($H_2O_2$ induced). HT1080 cell line was used.

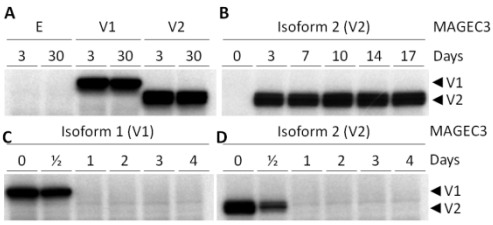



**Fig 4**. Doxycycline induced MAGEC3 expression of two isoforms at 3 and 30 days (A), Isoform 2 shows stable expression over constant induction (B). MAGEC3 is rapidly eliminated from the cell after removal of doxycycline for both isoforms (C,D).

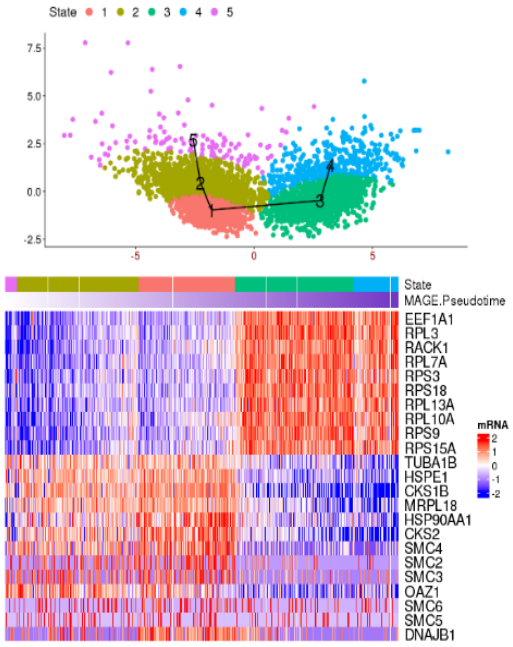
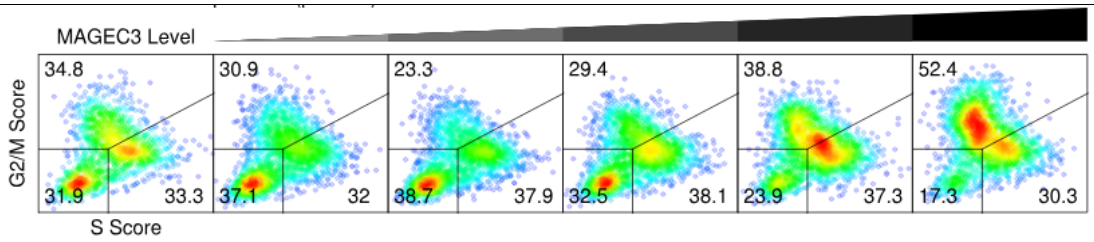



**Fig 5**. Exploratory single-cell sequencing in HT1080 cells with induced MAGEC3 expression. (Top) TSCAN induced minimum spanning tree and ICA space shows a bimodal MAGE expressing clusters. (Bottom) genes associated with MAGE expression (high MAGE to the left, low to the right) show association with SMC and stress genes



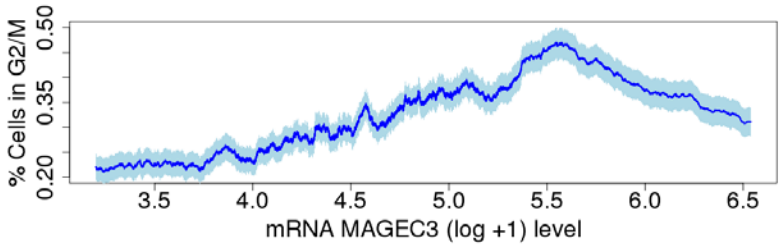




**Fig 6.** Single cell RNAseq-based (mRNA) demonstration that MAGEC3 is correlated with cells stalling in G2/M. (Right) A moving average estimate clearly shows the relationship between MAGEC3 and G2/M (N ~ 25,000 cells)

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

Regulatory plans.

- Based on findings so far, we will develop the mouse studies as originally planned. In the next reporting period we have budgeted time in our original SOW to handle regulatory filing locally and with ACURO.
- Now that our sequencing pipelines are ready, we will advance with human subjects exemption regulatory filings.

Based on the current data, we have the following **planned experiments.**

- We will plan experiments to test the mechanism of MAGEC3 protein silencing despite a pool of measurable mRNA. The usual suspects are proteasome and ubiquitination machinery. The BioID screen will be a back up approach.
- We will confirm the connection between SMC and MAGEC3 using co-IP and the BioID screen.
- We will execute DNA repair studies using labeled nucleosides (BrdU incorporation and repair) and direct measures of damage (H2AX-gamma).

Based on the completed and planned experiments so far, we have outlined two manuscripts that should be submitted in the next reporting period.

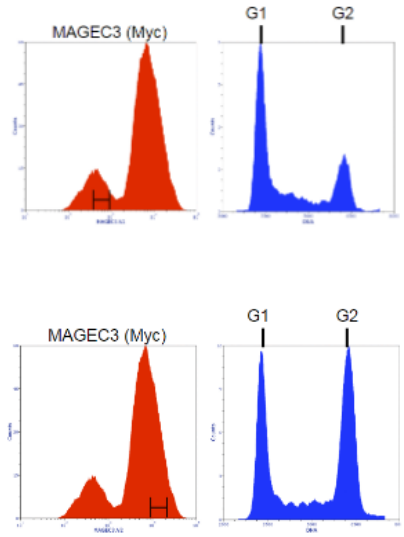



**Fig 7**. Cell cycle confirmation by flow cytometry. Myc tagged anti-MAGEC3 shows the two cell populations (left hand panels). Cell cycle classes by DAPI as a window of MAGE expression slides from left to right (right hand panels) implies higher expressing cells are more often in G2.

Manuscript 1. Tentative title: “MAGEC3 is a cell cycle related tumor suppressor gene”

- Design of inducible cell lines, validation by qPCR and western
- Growth and apoptosis
- Cell stress response
- DNA repair assays

Manuscript 2.

- Growth modulating MAGEC3 and p53 status.
- BioID screen to determine nominal and time-dependent protein interactions.
- Confirm with IP showing interactions.
- Evaluate connection with SMC complex hypothesis.

**IMPACT**

**What was the impact on the development of the principle discipline of the project?**

Nothing to report.

**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.

**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**

Our institute policy with respect to tumor sequencing is beginning to shift at a high level. Instead of investigators sequencing individual tumors from the biobank through our internal shared resources, the institute has started to organize sequencing through contractors arranged by the ORIEN consortium. This work is overseen by an additional institutional committee the Translational Research Group and requires additional approvals to prioritize tissue use on campus. This change in policy has led to a serious delay in filing our protocol for Non-Human Subjects Research exemption and HPRO for the sequencing portion of the work as we wait for the new protocols and re-consents to come online. To resolve this issue, our lab group is investigating the implications of the new policy and the quality of the subsequent data. If the genomic data are of sufficient quality, we will use the cohort of ovarian cancer patients sequenced in collaboration with ORIEN. We anticipate that the genomic data may not be incomplete and may not have the full range of RNA/DNA and methylation we need to study the silencing of our gene. If the data are sufficient, our institutional policy is that these samples represent data accessible without filling for exempt status (in the style of public databases such as TCGA or GTEx) and our planned HPRO submission will be sufficient.

**Changes that had a significant impact on expenditures**

Nothing to report.

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

Nothing to report.

**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.

**PRODUCTS**

**Publications, conference papers and presentations**
Nothing to report.

**Websites or other internet sites**
Nothing to report.

**Technologies or techniques**
Nothing to report.

**Inventions, patent applications and or licenses**
Nothing to report.

**Other products**
Cell lines. We have generated inducible (Tet-ON promoter) MAGEC3 lines in HT1080, PC3, and A-498 cell lines. The 786-O do not have an operator vector and express MAGEC3 constantly at the mRNA level.

**PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS**
**What Individuals have worked on the project?**

| | |
|---|---|
| **Name** | Kevin Eng |
| **Project Role** | PI |
| **Researcher Identifier** | |
| **Nearest person month worked** | 1.8 |
| **Contribution to project** | Dr. Eng is responsible for the overall direction of the project. He executes the statistical analyses for the project and oversees the genomics/informatics analyses. |
| **Funding support** | R01CA16333 (Co-I, 1%), R01CA1889900 (Co-I, 5%), R01CA205246 (Co-I 7%), R01CA172105 (Co-I, 2%). |

| | |
|---|---|
| **Name** | John Krolewski |
| **Project Role** | Co-investigator |
| **Researcher Identifier** | |
| **Nearest person month worked** | 1 |
| **Contribution to project** | Dr. Krolewski advises on cancer genetics and will supervise the animal portion of the project. |
| **Funding support** | P30CA016056 (Program Leader, 10%) |

| | |
|---|---|
| **Name** | Alexander Truskinovsky |

| | |
|---|---|
| **Project Role** | Pathologist |
| **Researcher Identifier** | |
| **Nearest person month worked** | 0 |
| **Contribution to project** | None. Dr. Truskinovsky was scheduled to be the pathologist for the project but has left the institute. |
| **Funding support** | None. |

| | |
|---|---|
| **Name** | Kayla Morrell |
| **Project Role** | Biostatistician |
| **Researcher Identifier** | |
| **Nearest person month worked** | 2 |
| **Contribution to project** | Ms. Morrell performed data management and cleaning for the project and assisted in experimental design and data analysis. She left the project in August 2018. |
| **Funding support** | None. |

| | |
|---|---|
| **Name** | Seyedeh (Mojy) Shourideh-Ziabari |
| **Project Role** | Research associate |
| **Researcher Identifier** | |
| **Nearest person month worked** | 5 |
| **Contribution to project** | Ms. Shourideh-Ziabari performed bench experiments (cell culture, transfections and nucleic acid work). She has left the project as of December 2018. |
| **Funding support** | None. |

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

Ms. Morrell, Ms. Shourideh-Ziabari and Dr. Truskinovsky have moved on to other roles or left the institute. We have decided to replace Ms Shorideh-Ziabari with a doctoral level researcher. At this point we have made an offer to a postdoctoral scholar to start in July/August 2019. Biostatistics needs will be met by Dr. Eng in the meantime with the option to purchase time from masters level statisticians from the institutional core facility. Pathology needs will be met by Dr. Truskinovsky's to be named replacement as institutional gynecologic cancer pathologist.

**What other organizations were involved as partners?**
Nothing to report.

**SPECIAL REPORTING REQUIREMENTS**
None

**APPENDICES**
None