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

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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

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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	Complete
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	Complete
Sub Task 2. Construct lentiviral MAGEC3 lines	13-18	Complete

Sub Task 3. Characterize cell lines' proliferative phenotype and MAGEC3 expression. Perform statistical analyses.	18-30	Partial – in 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	Complete
Sub Task 3. Perform xenograft studies, isolate and collect tumors. Conduct statistical analyses.	24-36	Partial – In progress
Major Task 5 is to prepare manuscripts for submission		Partial – in progress

What was accomplished under these goals?

Accomplished under major task 1.

Major Task 1 was completed in previous reporting period.

Accomplished under major task 2

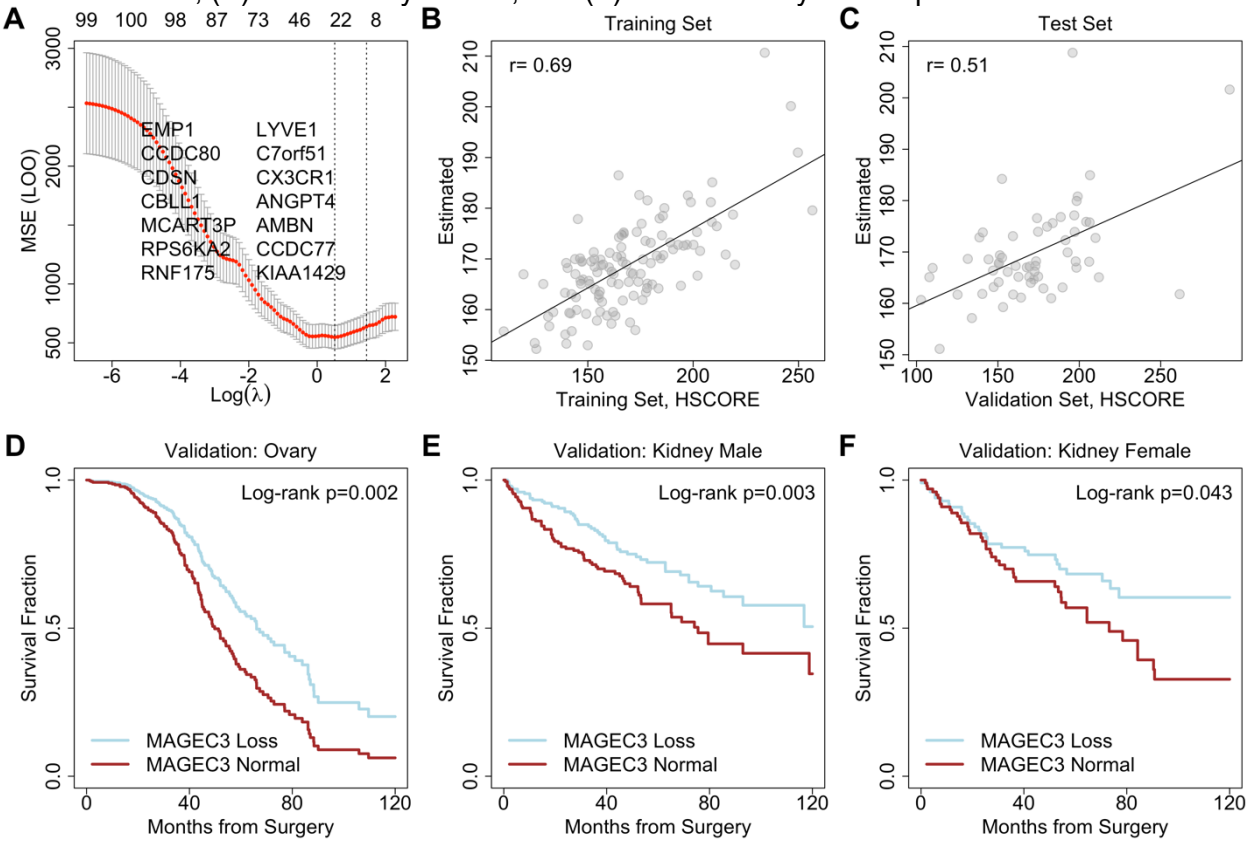
We previously reported the analysis of N=467 cases stained on an institutional tissue microarray and their clinical correlates.

In this reporting period, we conducted a genomic analysis of MAGEC3 correlates in the mRNA space.

As part of our planned study to assess the clinical relevance of MAGEC3, we procured MAGEC3 protein expression in normal and tumor patient tissue samples and assessed its association with various clinicopathologic features as well as its impact on patient survival. When dichotomized at the median, we found that tumor expression of MAGEC3 fell into two categories – those that are significantly lower than normal tissue expression (loss) and those that are no different from normal tissue expression (normal). In our ovarian cancer cohort (N=394), we found that normal expression of MAGEC3 was associated with cases ascertained after 2006 (OR=5.86, 95%CI: 3.8-9.3, $p<0.001$). Additionally, loss cases had a much more favorable survival trend (HR=0.71, p=0.004). In our kidney cancer cohort (N=220), we found that normal expression was associated with black patients (OR=6.70, 95%CI:1.8-47.6, p=0.003) and also with non-clear cell (papillary) histology (OR=14.5, 95%CI: 4.1-99.5, $p<0.001$). Interestingly, a MAGEC3-sex interaction was observed while investigating the impact of expression on survival. Cases with MAGEC3 loss had significantly better survival trends in males (HR=0.48, p=0.01) but an insignificant effect in females (HR=1.81, p=0.3).

In an independent cohort (N=180) containing both MAGEC3 protein levels and RNA sequencing data, we built a machine learning model to predict MAGEC3 protein expression. This model was tested in a fourth, independent cohort with transcriptomic data (N=282 ovary and N=606 kidney). Results of survival analyses performed using these predicted protein scores validated those discovered in our original ovary and kidney datasets (**Figure 1**).

Figure 1. RNA-based predictive model for MAGEC3 expression. (A) Leave out one (LOO) cross-validation error for tuning parameter selection. Fitted values in the (B) training set and (C) withheld test set. Survival estimate validation in the independent pan-TCGA dataset for (D) ovarian cancer, (E) male kidney cancer, and (F) female kidney cancer patients.

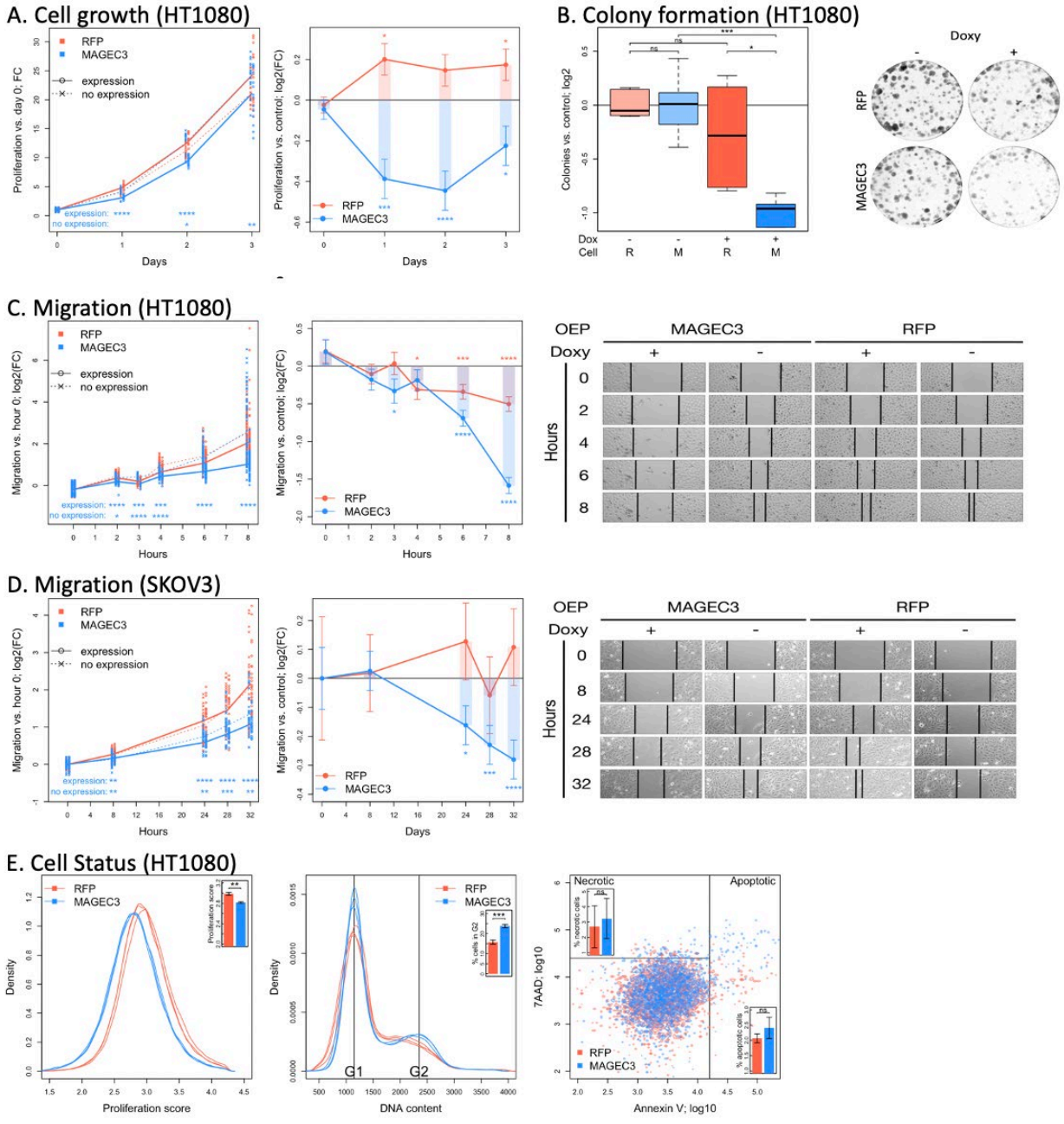


Accomplished under major task 3

As we previously reported, due to cell lines having undetectable MAGEC3 levels, we designed a doxycycline inducible system to express MAGEC3 (or RFP for control) in cells. In vitro growth assay showed that cells overexpressing MAGEC3 reduced cell growth compared to cells overexpressing RFP (Figure 2A). MAGEC3 also reduced 2D colony formation (Figure 2B) and migration (Figure 2C-D). Using flow cytometry analysis we found that MAGEC3 reduces cell proliferation and stalls cells in G2 cell cycle phase, without any effect on cell death (Figure 2E).

Figure 2. MAGEC3 is a tumor suppressor gene. Cells were infected with MAGEC3 (or RFP) under doxycycline inducible promoter. All experiments were done in the presence or absence of doxycycline. (**A**) MTS assay for in vitro growth curves (left panel) and normalized growth to cells not expressing RFP or MAGEC3 (right panel) are shown (n = 16 replicates from 2 independent experiments; left panel: Student's t-test was used to compare MAGEC3 to RFP cells; right panel: linear regression was used to compare cells overexpressing the protein of interest to cells that do not). (**C**) 2D colony formation showing the area of colonies after 10 days of MAGEC3 expression (left panel). Representative images are shown in the right panel. (n = 3 replicates; Student's t-test was used for comparisons). (**C-D**) Wound healing assay for

migration; left panel shows cell migration over time and middle panel the relative migration levels compared to cells not expressing the protein of interest. Representative images are shown in the right panel. (n = 28-41 measurements from 3-5 replicates from 2 independent experiments for panel C and n = 29-30 measurements from 3 replicates for panel D; left panel: Student's t-test was used to compare MAGEC3 to RFP cells; right panel: linear regression was used to compare cells overexpressing the protein of interest to cells that do not). (**E**) Flow cytometry analysis for proliferation (left panel), cell cycle (middle panel), and cell death (right panel) of cells expressing MAGEC3 or RFP for 2 days (n = 3 replicates each with more than 75000 cells; Student's t-test was used to compare MAGEC3 to RFP cells).
** p ≤ 0.05 | ** p ≤ 0.01 | *** p ≤ 0.001 | **** p ≤ 0.0001.*

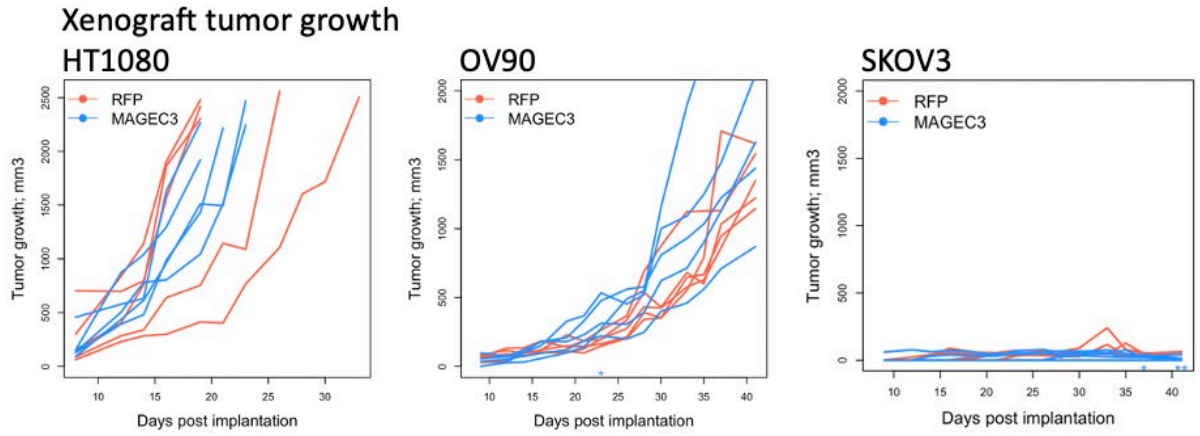


Accomplished under major task 4

IB studies were determined to be infeasible and favored xenograft studies as proposed in our alternative approach.

We implanted 1 million cells in the right flank of NSG mice and animals were treated with doxycycline (administered with their food). Tumor burden was measured and once tumor size reached 2000mm3 (institutional end point) animals were sacrificed and tumor tissue was collected. Once all tumor tissues are collected we will quantify MAGEC3 and RFP (both of them are myc tagged for easy detection) by western blot analysis to confirm that cells express MAGEC3 (or RFP). Animals with tumors that do not express the protein of interest will be removed from the study. This experiment is still ongoing.

Figure 3. MAGEC3 effect on tumor growth. Cells expressing MAGEC3 or RFP (control) under a doxycycline inducible promoter were implanted in the right flank of NSG mice. Tumor size was measured using electronic calibers 3 times per week (n = 5 per group).



Accomplishments under major task 5

In this reporting period, we prepared the manuscript entitled “Prognostic machine learning model of MAGEC3 in ovarian and kidney cancers” for submission. The goal of this paper was to establish the clinical relevance of MAGEC3. We quantified normal and tumor protein expression of MAGEC3 via immunohistochemistry in N=394 ovarian cancers and N=220 renal cell carcinomas, assessed the correlation of these values with clinicopathologic features, and modeled survival using univariate and multivariate models. We found that MAGEC3 protein was sporadically lost in ovarian cancers, with 50% of cases falling below the 9.5th percentile of normal tissue expression. Cases with MAGEC3 loss demonstrated better overall survival in both ovarian cancers [HR=0.71, p=0.004] and male renal cancers [males: HR=0.48, p=0.01; females: HR=1.81, p=0.3] similar to patients with germline or somatic BRCA2 loss. To extend these results, we considered N=180 independent cancers of various types and built a RNA-based model of protein expression. This model was tested in a fourth, independent cohort with transcriptomic data (N=282 ovary and N=606 kidney). Results of survival analyses performed using these predicted protein scores validated those discovered in our original ovary and kidney datasets, indicating that MAGEC3 protein is a prognostic biomarker. To elucidate the function of

MAGEC3, we performed integrative genomic analysis and observed an association between MAGEC3 protein and genes affecting stress response, including those involved in cell cycle and DNA damage repair. Additionally, it is correlated with tumor mutational burden in patients with mutated oncogenes. These associations suggest that MAGEC3 protein levels may be used to identify patients with deficient DNA damage repair mechanisms that can be targeted by PARP inhibitors. Thus, using our machine learning model to predict MAGEC3 protein levels from RNA sequencing data can facilitate the identification of patients for treatment stratification according to their MAGEC3 status.

A previous version of this manuscript was archived in the preprint server medRxiv (doi: https://doi.org/10.1101/2021.04.30.21256427). We are currently in the process of submitting an updated version to peer-reviewed journals for publication.

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 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 – 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.

In a retrospective cohort study, we determined that, unlike other MAGE family members, the MAGEC3 protein is normally expressed in ovarian tissue but is lost in half of ovarian cancers. Similar to other predisposition genes like BRCA2, survival modeling suggests that expression

loss is associated with favorable progression-free survival and continued expression is associated with response to platinum therapy. Using transcriptomic modeling, we predicted that MAGEC3 expression is associated with stress related cell cycle stalling and DNA repair pathway expression.

We reported that MAGEC3 was difficult to measure by bulk RNA sequencing and subsequently developed an RNA-based linear model to predict MAGEC3 protein levels learned from labelled data. The accuracy of the predictor was evaluated in a validation set and subsequently applied to ovarian cancer patients within the TCGA pan-cancer dataset. Using the predicted scores, we were able to validate the results found for cases with directly measured MAGEC3 scoring. The ability to accurately predict protein levels based off an RNA predictor greatly increased the sample size and impact of our results.

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 (in the previous reporting period). We returned to 50% operational staff in June 2020 and have remained at this level of staffing to the present date. The institution reports that they will reassess staffing levels during Summer 2021.

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.

6) Products

Journal publications
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

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.

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?
Nothing to report

What other organizations were involved as partners?
Nothing to report

8) Special reporting requirements
Nothing to report

9) Appendices

Award Chart

OC170368: Novel Tumor Suppressor Gene in Hereditary X-linked Ovarian Cancers

PI: Kevin Eng, Roswell Park, NY

Budget: $773,100

Topic Area: OCRP

Mechanism: Funding Opportunity

Research Area(s): Ovarian Cancer, Cancer genetics

Award Status: 15 May 2018-14 May 2021

Study Goals:

To strengthen the evidence for or against heritable ovarian cancer X-linkage in an candidate locus and to conduct detailed functional studies of the candidate gene.

Specific Aims:

Aim 1 is to determine whether MAGEC3 expression is silenced at the RNA or DNA level using deeper sequencing in familial ovarian cancers seen in women with an affected paternal grandmother from the FOCR, consistent with the TSG hypothesis.

Aim 2 is to test the hypothesis that restoration of MAGEC3 expression can suppress the tumor phenotype *in vitro*.

Aim 3 is to test the hypothesis that orthotopic mouse models can recapitulate the tumor suppressor effects of MAGEC3.

Key Accomplishments and Outcomes:

Publications: none to date

Patents: none to date

Funding Obtained: none to date