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14. ABSTRACT Prostate cancer disproportionately affects African American men and our laboratory has determined that an at-risk polymorphism in the gene for SELNOF is 10-times more frequent in the genomes of African Americans and this genetic variation is likely to result in lower SELENOF levels in tissues. Consistent with these observations is data indicating that the levels the SELENOF protein are lower in the prostate tumors of African American men as well. It remains possible that reduced levels of SELENOF are not contributing to cancer progression, but are just a "bystander" to the changes that occur during malignancy. The most significant finding during the initial funding period was that the reduction of SELENOF in immortalized prostate epithelial cells using ShRNA argues against that possibility. The reduction of SELENOF in these cells altered the morphology of the cells, increased their ability to grow in soft agar and enhanced their ability to migrate in a scratch assay, all of these are hallmarks of malignant transformation. These data therefore provide solid evidence that the loss of SELENOF is mechanistically linked to prostate cancer aggressiveness and contribute to the disparity in disease outcome experienced by African Americans.

15. SUBJECT TERMS
Prostate cancer, health disparity, tumor suppressor

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1. Introduction

Prostate cancer is the second leading cause of death among American men and the disease is disproportionally greater among African American men who experience the highest incidence and mortality from PCa world-wide as compared to other racial groups in the US. The identification of risk factors that predominate among African American men is a critical step in reducing the risk of dying of PCa in that population, and may lead to identifying risk factors in the US male population at large. It is hypothesized that the loss of SELENOF protein plays a role in the disparity in PCa incidence and outcome between African American and Caucasian men and our broad goal is to determine this role. Among the data supporting this hypothesis are previous results indicating 1) the dramatic reduction of SELENOF in prostate tumors compared to adjacent benign tissue [1], 2) an association between specific *SELENOF* alleles and the risk of getting prostate cancer or dying from the disease [2], 3) a 10-fold higher frequency of the at-risk allele in African Americans [1] and 4) lower levels of SELENOF in prostate cancers from African Americans as compared to Caucasians [1]. The proposed studies included genetically engineering human prostate derived cells to over- and under-express SELENOF to interrogate mechanistically the consequences of its activity. Human tissues will be examined, both as tissue microarrays and formalin fixed, to determine associations between race, SELENOF genotype and levels, selenium levels and clinical parameters of prostate cancer. An animal model for the impact of the loss of SELNOF on prostate carcinogenesis will be developed by breeding asymptomatic SELENOF knock-out mice with mice that that develop prostate cancer. Collectively, the investigation of the impact of the reduction of SELENOF levels on prostate cancer is anticipated to generate new information about the disease and the disparity in incidence and mortality experienced by African American men.

2. Keywords

Prostate; cancer; selenoprotein; polymorphism; disparity; cell culture; tissues; mouse models; regulation; transcription; selenium

3. Accomplishments

- **What were the major goals of the project?**

Below are the aims presented in the awarded grant:

Aim 1. Determine the differences in levels of SELENOF between African American and Caucasian men and establish whether there is an association between SELENOF serum and tissue levels and clinical parameters including PSA levels, tumor sage and grade, and outcome.

Aim 2. Determine whether the absence of SELENOF in the prostate reduces the time to the appearance of prostate cancers, the incidence of these tumors, and their severity in mouse models genetically engineered to develop prostate cancer.

Aim 3. Determine the mechanism by which reduced SELENOF levels contribute to a higher prostate cancer risk and poorer clinical outcome. We will reduce the levels of SELENOF in immortalized and primary human prostate epithelial cells as well as increase SELENOF in human tumor cell lines cell lines. These derivative cells will be examined for features associated with the transformed phenotype.

- **What was accomplished under these goals?**

Aim 1: We obtained the "120 Case High Grade Race Disparity TMA" from the Prostate Cancer Biorepository Network (PCBN). These TMA is specifically designed for comparing biomarkers in African American and Caucasian patients. Being enriched for higher grade cases, the TMA includes 60 cases of tumor and normal tissue from each group matched on age, grade and key clinical and demographic data across 3 blocks. Staining optimization was performed using "practice slides" obtained from the Pathology Department for both SELENOF and E-cadherin, the latter being a marker for staining of the outer membrane. Following optimization, the 3 slides were stained with the designated antibodies, yielding high quality images. Even though the Vectra® automated multispectral imaging system can distinguish epithelial cells from surrounding tissue, each of the 1,200 cores were individually examined to confirm the designations. Signals for the entire cell, the nucleus and outer membrane were quantified on an individual

cell basis and the information assimilated into a data file. This file has been provided to a statistician (Dr. Liu) who is currently performing the analysis.

As part of the goals indicated for Aim 1, we are obtaining archived prostate tissues from African American and Caucasian men from which we will determine 1) levels of SELENOF, 2) SELENOF genotype and 3) selenium levels. Each tissue needs to be recovered from the UIC tissue bank, demographic information collected, examined by a pathologist to determine benign and cancerous sections for collection and individual "pieces" of tissue collected for preparation of DNA for genotyping and another segment for selenium analysis. To date, 50 tissue samples have been collected in this manner. We have decided to store the blocks until all of the samples have been collected so that they can all be processed at the same time for immunohistochemistry. For several tissues, we have sent multiple segments from the same tissue to our collaborator John Brockman at the University of Missouri Research Reactor Center for selenium analysis by Instrumental Neutron Activation Analysis and we were very satisfied with the reproducibility of the obtained analyses.

Aim 2: The planned animal studies involved the mating of SELENOF knockout mice to either mice that get prostate cancer due to the over-expression of the c-myc gene (Hi-Myc) or due to the loss of the PTEN tumor suppressor. All of the required mouse strains have been obtained, methods for genotyping were established and cross breeding of strains has begun following a set of setbacks described below.

Aim 3: The goal of this aim to alter the levels of SELENOF in tissue culture cells to determine the consequences on a host of transformation-related parameters. Such functional studies are critical in distinguishing a contributing role for (the loss of) SELENOF in cancer progression from a bystander effect.

<u>Reduction SELENOF in immortalized prostate epithelial cells</u>. The RWPE-1 prostate-derived cell line is immortalized but does not exhibit the transformed phenotype. To recapitulate what happens in prostate cancers that exhibit a loss of SELENOF, the levels of SELENOF were reduced using a commercially available shRNA construct. The reduction in SELENOF was verified by both western blotting of protein extracts and confocal microscopy. As seen in Figure 1 below, RWPE-1 cells transfected with the shRNA expressed reduced SELENOF levels compared to either naïve RWPE-1 cells or the same cells transfected with a control scrambled shRNA construct.

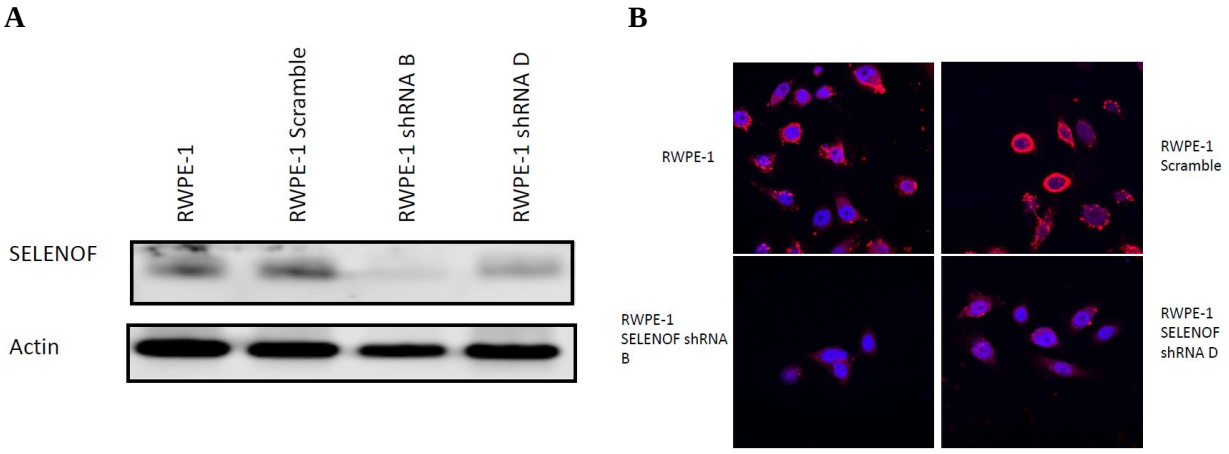

Figure 1. Effective knock-down of SELENOF in RWPE-1 immortalized epithelial cells. A. Western blot indicating the levels of SELENOF in native RWPE-1 cells, those cells (RWPE-1), transfected with a scrambled shRNA construct (RWPE-1 Scramble), and 2 different knock-down clones ((RWPE-1 SELNOP shRNA B and D). B. Immunofluorescence indicating knock-down of SELENOF, comparing RWPE-1 cells transfected with the scrambled control and the SELENOF shRNA construct (two different clones).

One of the hallmarks of transformation is the ability of cells to grow in soft agar. RWPE-1 cells do not grow when suspended in soft agar and cells expressing reduced SELENOF did not exhibit altered growth kinetics. However, reducing SELENOF resulted in the dramatic increase in the cells ability to grow in soft agar (Figure 2).

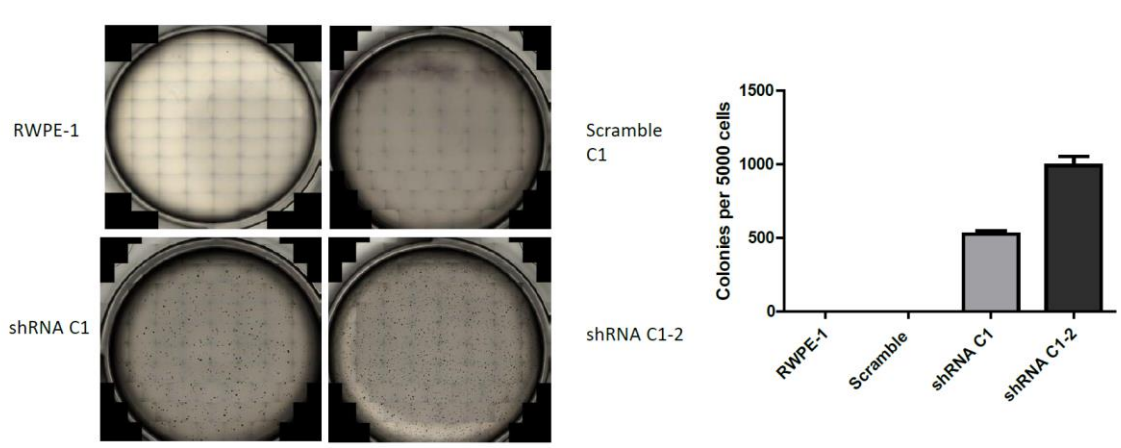

Figure 2. Reducing SELENOF in RWPE-1 cells results in the acquired ability to grow in soft agar. A. Representative plates indicating that SELENOF knock-down cells form colonies when plated in soft agar as compared to either native or cells transfected with a scrambled control construct. B. The data from 3 independent experiments is presented graphically as the number of colonies per 5000 cells plated.

Functional polymorphism in SELENOF are associated with an increased risk of dying from prostate cancer [2]. One of the features of aggressive, metastatic cancer is cell invasion, which is related to cellular migration that can be measured using the simple and reproducible "scratch wound healing assay" in which cell motility into a "gap" in the monolayer is assessed. As seen in Figure 3, there is little mobility in the "gap" by control cells and significantly more cells in that region in the knock-down cells.

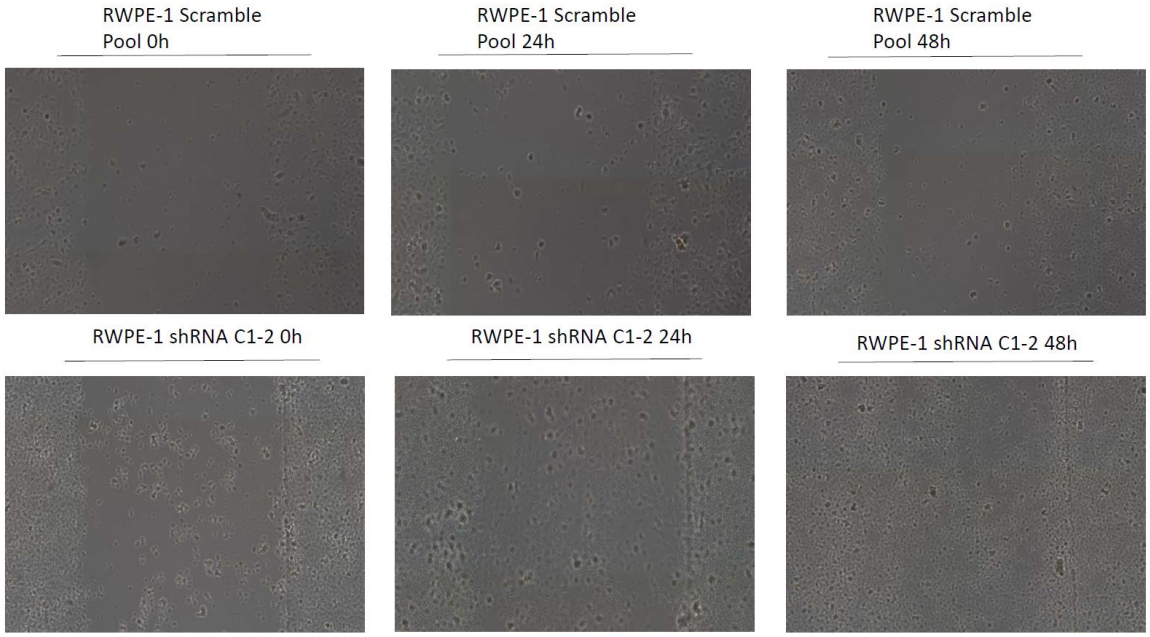

Figure 3. Knocking down SELENOF is RWPE-1 cells enhances their ability to migrate into a cell free zone as compared to cells transfected with a control scrambled construct.

The prostate is a highly specialized organ among whose functions is to accumulate and secrete large amounts of citrate as a component of semen, thus supporting sperm health. Zinc accumulation in the prostate inhibits the mitochondrial enzyme aconitase that otherwise would convert citrate to isocitrate and into the Krebs cycle for the generation of ATP by oxidative phosphorylation. Energy requirements of the organ are instead provided by a reliance on energy-inefficient aerobic glycolysis [3]. One of the hallmarks of prostate cancer is the alteration of this process where zinc levels decline dramatically, relieving the inhibition of aconitase. As a result, re-entry of citrate in the pathways that provide both energy (Krebs cycle/aerobic oxidation) and cellular building blocks (lipogenesis) in support of cancer growth [3]. Therefore, the metabolic changes that typically occur during prostate carcinogenesis are

quite different than those that occur during the evolution of other solid cancer types. Most solid tumors undergo a shift from oxidative phosphorylation as the primary energy source in the normal tissue to a heavy reliance on oxidative glycolysis in the cancer. This phenomenon was first recognized by Otto Warburg in the 1920s and has been a focus of cancer biologists ever since.

The effect of reducing SELENOF in RWPE-1 cells on mitochondrial respiration was determined using a Seahorse XF analyzer that measures oxygen consumption rate and various related parameters following the use of specific electron transport chain inhibitors (Figure 4). As seen in the figure, reduction of SELENOF significantly enhanced mitochondrial respiration as indicated by the significant increase in the oxygen consumption rate (OCR).

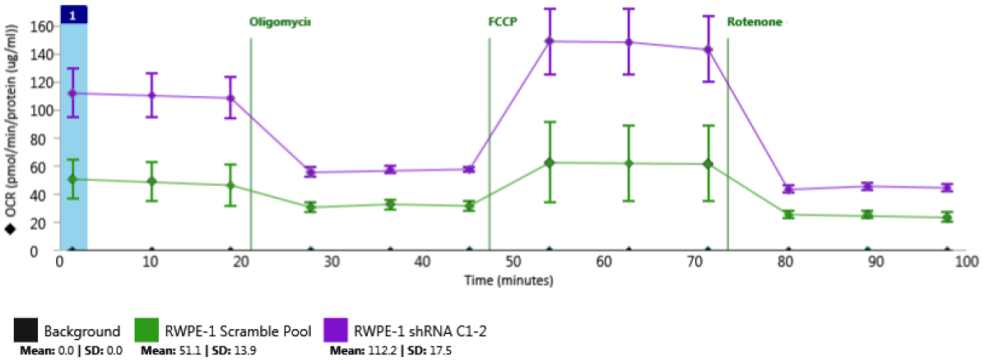

Figure 4. Knocking down the level of SELENOF in RWPE-1 cells results in in a dramatic increase in oxygen consumption as determined using the Seahorse XF analyzer.

The significance/impact of these data are addressed below.

Mechanism of repression of SELENOF in prostate cancer. SELENOF levels are consistently lower in prostate cancer compared to adjacent benign tissue [1]. We have also reported that SELENOF levels are lower in the prostate cancers of African Americans compared to Caucasian men. As part of the effort to understand these observations, we determined that although there was much less SELENOF in immortalized prostate cells compared to cell lines derived from prostate cancers, the levels of SELENOF mRNA were very similar (Figure 5). In addition, we have generated SELENOF expression constructs that have resulted in SELENOF being produced in breast cancer-derived cells but not prostate cancer cells. These data indicate that there may be a post-transcriptional mechanism of SELENOF regulation relevant to prostate cancer etiology.

A. B.

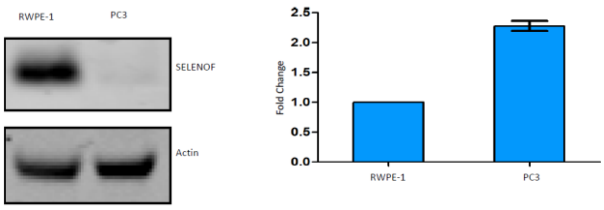

Figure 5. SELENOF levels are much higher in immortalized RWPE-1 cells (A) but there is slightly lower levels of SELENOF mRNA in those cells (B). Data in B is from 3 biological replicates.

SELENOF is a member of the selenoprotein family of peptides that include selenium in the form of selenocysteine, encoded by an in-frame UGA "stop" codon [4]. This codon is recognized during translation as selenocysteine due to a regulatory region in the 3'-untranslated region referred to **Se**leno**c**ysteine **I**nsertion **S**equence (SECIS). The SELENOF SECIS element is unusual as it contains two SECIS elements [4]. We have hypothesized that the decoding of the UGA codon contributes to the observed differential protein levels. Towards resolving this issue, we have generated SELNOF expression constructs containing each SECIS element as well as both, and also generated reporter constructs in which a TGA was introduced in the reading frame of firefly luciferase gene and "readthrough' can be monitored with each individual SECIS element and both inserted downstream.

- **What opportunities for training and professional development has the project provided?**

Dr. Elhodaky is an M.D. and Ph.D. student supported by the DOD award. He has presented his research to the "Works in Progress" seminar series and an oral presentation at the Nutrition 2019 conference hosted by the American Society

of Nutrition in Baltimore, MD in June. He attends the weekly GU Tumor Board meeting in the College of Medicine, has participated in the Ingenuity Pathway Analysis workshop, UIC Research Informatics Core, 01/2019, Science Writing and Medical Communications, CHIentist, 05/2019 and has been accepted to participate in the ComSciCon workshop at Northwestern University in August of 2019. In addition to Dr. Elhodaky, an undergraduate student (Shrinidhi Kadkol) has participated in this project, becoming adept at molecular cloning and analysis.

- **How were the results disseminated to communities of interest?**

Our research on SELENOF was included in a review currently "In Press" in Biological Trace Element Research' with DOD support acknowledged. Our progress was presented to the Pathology Department's "Works in Progress" seminar series, and as a seminar to the University of Illinois Cancer Center's Translational Oncology seminar series on July 11th.

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

Specific Aim 1: We will perform the analysis of the data that has already been collected from the staining of the ethnicity TMA and report on the results, or consider using that data as part of a more comprehensive manuscript that includes results from the other aims. In addition, we will continue to collect the rest of the clinical samples from the UIC tumor bank, have the pathologist review each sample and move forward on their processing for SELENOF localization and quantification, genotyping and selenium analysis.

Specific Aim 2: Breeding of the SELENOF mouse to both prostate cancer mouse models (PTEN +/- and Hi-myc) and collect prostate tissue for analysis as indicated in the proposal.

Specific Aim 3: We will continue to examine the phenotype of human prostate-derived cell lines engineered to over- or under-express SELENOF. We will also continue to examine the mechanism by which SELENOF levels are reduced in cancer and in the tumors of African American men as compared to Caucasian men, focusing on perturbations in the translation of SELENOF mRNA, based on the reported progress.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**

Prostate cancer is a very common source of morbidity and mortality among men in the United States and across the world. The data obtained during the first year of DOD funding for the first time established that the loss of SELENOF is a likely contributor to prostate cancer progression and not a mere bystander where its loss is a consequence, not a cause of the disease. This was established using tissue culture cells where SELENOF levels were reduced using a shRNA and consequentially the cells gained the ability to grow in soft agar and migrate in culture, two parameters associated with aggressive cancer. In addition, the data obtained also provided evidence that the loss of SELENOF may be contributing to unusual shift in energy metabolism that occurs during prostate cancer progression from an inefficient oxidative glycolytic mechanism that promotes citrate production to one that instead provides the energy and building blocks required for cancer growth. Collectively, these accomplishments reveal a new and significant aspect of prostate cancer and establish SELENOF as a likely prostate cancer tumor suppressor.

- **What was the impact on other disciplines?**

Based on the data obtained from our investigation into the mechanism by which SELENOF levels are reduced in prostate cancer indicating that post-transcriptional mechanisms are likely involved, we have identified a likely translational control of SELENOF that may involve the translation factor EIF4a3. Among its other functions, EIF4a3 is induced in times of low selenium availability and suppresses the translation of several selenium containing proteins known to be sensitive to selenium status by binding to the SECIS element of the RNAs encoding those selenoproteins [5]. There is a structurally similar binding site in the SECIS element of SELENOF, which include the polymorphism associated with prostate cancer mortality and differently represented among Africa Americans. Collectively, these

data may have identified a novel mechanism of regulating selenoproteins that is influenced by both diet (selenium) and genetics. These results will have a very significant impact on the field of selenium biology in general.

- **What was the impact on technology transfer?**
  Nothing to report

- **What was the impact on society beyond science and technology?**
  Health disparity is a significant issue facing society with many factors contributing to the increased risk of aggressive prostate cancer and dying from the disease affecting African American men. Current evidence supports the conclusion that there are genetic factors that account, at least in part, to these circumstances. Our efforts supported by the DOD are contributing to the discovery of one such genetic factor: a functional polymorphism in the SELENOF gene that is approximately 10 times more prevalent in African Americans and is associated with the risk of dying from prostate cancer [1,2]. Understanding the mechanism by which this naturally occurring genetic variation increases the risk of suffering from prostate cancer is hoped to help to identify those at greatest risk so that increased surveillance and better care can be provided, as well as potentially identify new targets for therapy, to help reduce the burden of prostate cancer on the African American population.

5. Challenges/Problems

- **Changes in approach and reasons for change.**

  The only significant change in the approach was to investigate the translational regulation of SELENOF. This change was initially due to the observation that the levels of SELENOF levels are lower in prostate cancer and there were dramatic differences in SELENOF protein levels in tumor-derived human cell lines as compared to immortalized prostate epithelial cells. Subsequently it was determined that expression of our SELENOF expression constructs resulted in minimal or no increase in the corresponding protein. To investigate this phenomenon, which may reveal why SELENOF levels decline in prostate cancer, we have generated several new expression constructs that are supported by combinations of 3'-regulatory elements as well as reporter constructs in which the efficiency of these elements can be measured by the readthrough of introduced in-frame UGA triplet in the luciferase reporter gene.

- **Actual or anticipated problems or delays and actions or plans to resolve them.**

  The major problem encountered during the first year of funding involved the proposed animal studies. The essential strain, the SELENOF knockout mice, arrived from the supplier positive for Helicobacter and mouse Norovirus. As a result, the mice had to be rederived by breeding the infected mice by breeding in isolation and transferring pups to surrogate mothers. This was performed successfully. However, genotyping the offspring indicating that heterozygotes (hemizygotes) and not homozygote SELENOF knockout mice were provided. Breeding of heterozygotes ultimately yielded SELENOF null mice and this colony is currently being expanded.

  The Hi-Myc mice we obtained were similarly infected with Helicobacter and likewise had to be rederived. Breeding of the surrogate reared mice yield offspring with a normal phenotype, however subsequent breeding yield litters in which approximately 50% of the pups demonstrated a severe leg anomaly. Breeding the pups that appeared normal from the mixed litter again yielded 50% deformed pups. That colony was eliminated and we sought to obtain Hi-Myc mice from an alternative source. We obtained the appropriate MTA to the NIH to allow the transfer of Hi-Myc mice from a colony at the Illinois Institute of Technology and that MTA was only recently signed off on and we are awaiting the transfer of the mice.

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

  We decided to collect all of the tissues proposed for analysis so that they can be processed (tissue processing, imaging, selenium analysis and DNA preparation) to reduce experimental variability.

- **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

6. Products

- **Publications, conference papers and presentations.**
  - **Journal Publications.**

Diamond A.M. Selenoproteins of the Human Prostate: Unusual Properties and Role in Cancer Etiology. Biol..Trace Elem. Res. doi: 10.1007/s12011-019-01809-0. [Epub ahead of print] PMID:31300958, 2019. DOD support acknowledged.

- **Books or other non-periodical, one time publications.**
  Nothing to report
- **Other publications, conference papers, and presentations.**

"Selenoproteins and the Disparity in Prostate Cancer Experienced by African American Men"
Presented at a Translational Oncology research seminar, University of Illinois at Chicago Cancer Center in July, 2019.

- **Website(s) or other internet sites(s)**
  Nothing to Report

- **Technologies or techniques**
  Nothing to report

- **Inventions, patent applications, and/or other licenses**
  Nothing to report

- **Other products**
  Nothing to report

7. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**

Dr. Alan Diamond
Role: Principal Investigator
Months worked: 1.8
Contribution to project: Overall supervision and training, assured compliance with regulatory matters.

Dr. Maarten Bosland
Role: Qualified Collaborator
Months worked: 1.2
Contribution to project: Assured compliance with regulatory matters pertaining to animal studies, procured the mice, trained other participants in handling mice, including breeding and genotyping.

Dr. Andre Balla
Role: Co-Investigator
Months worked: 0.6
Contribution to project: Tissue procurement and histopathology

Dr. Mostafa Elhodaky
Role: Graduate Student:
Months worked: 12
Contribution to project: Molecular studies and tissue analysis

Michael Schlicht
Role in project: Technician
Months worked: 3
Contribution to project: Maintenance of animal colonies, mating and genotyping. Participation in molecular studies.

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

  Dr. Maarten Bosland is now receiving 3% effort on R37 CA227101, Defining the role of isoprenylated xanthones from the mangosteen for enhancing degradation of full length and variant forms of the androgen receptor, J. Johnson, P.I. and 3% effort on 1 U19AT010827, Translational biological signatures of resilience in postmenapause, Dr. Pauli, P.I.

  Dr. Diamond is now receiving 1% effort on 1RO1 HL1336622, The role of erythrocyte mitochondrial retention in sickle cell disease, Dr. Rivers, P.I.

  Dr. Kajdacsy-Balla's support for 3 grants (PC141050P1, 5RO1CA193497-03 and 1 RO1 EB009745B) have closed.

- **What other organizations were involved as partners?**

  Nothing to report

8. References

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