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TITLE: Contribution of SELENOF to the Disproportionate Mortality Experienced by African American Men Due to Prostate cancer

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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. 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 this funding period is that there is a block to the translation of SELENOF in prostate cancer cells. SELENOF expression constructs that failed to generate SELENOF in prostate cells were able to support the production of SELENOF from an inducible promoter in a different cell type. MCF-7 human breast cancer cells engineered to over-expressed SELENOF. These cells exhibited the opposite effects of prostate cells in which SELENOF levels were reduced. The parameters examined included growth rate, anchorage independent growth and oxygen consumption. These results contribute to the assignment of SELENOF as a tumor suppressor and support the hypothesis that the genetics of SELENOF is a factor in the disparity in prostate cancer mortality 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 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 results indicating 1) the dramatic reduction of SELENOF in prostate tumors compared to adjacent benign tissue, 2) an association between specific *SELENOF* alleles and the risk of getting prostate cancer or dying from the disease, 3) a 10-fold higher frequency of the at-risk allele in African Americans and 4) lower levels of SELENOF in prostate cancers from African Americans as compared to Caucasians. 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: In the initial reporting period, Tissue Microarray slides were obtained from the Prostate Cancer Biorepository Network (PCBN), staining with SELENOF was optimized, the slides were stained, and the signals for the entire cell, nucleus and outer membrane were quantified using the Vectra® automated multispectral imaging system. During the last year, these data were analyzed by a statistician yielding the following results for all tissues examined:

- Levels of SELENOF were significantly lower in prostate cancer compared to benign tissue irrespective of cellular location ($p<0.001$).
- No significant differences in cellular localization, the nuclear to cytoplasm ratio or the membrane to cytoplasm ratio was detected between cancer and adjacent benign tissue.

The obtained TMA was specifically designed to detect differences between prostate cancers derived from African American and Caucasian men. Race specific results were as follows:

- SELENOF levels were higher in all cellular compartments in the cancer tissues obtained from African American men than tissues obtained from Caucasian men, but there was no difference in the distribution of SELENOF in the cellular compartments examined in either cancer or benign tissue using two-sample t-tests to assess racial differences. This result was different than that we obtained with a previous TMA, and this is likely due to the difference in the samples includes in the TMAs: the earlier was comprised on samples representative of tissue with a lower clinical grade than the one we obtained from the PCBN.

- The difference in cancer vs. benign SELNOF staining was significantly associated with a higher grade of prostate cancer (Gleason score) in tissue derived from Africans American men, while that association was not found among Caucasians.

Part of the first aim was to obtain archived prostate tissue from African American and Caucasian men, collect demographic information, and have a pathologist examine the tissues to distinguish cancerous and benign portions for DNA, selenium and SELENOF analysis. Our goal was to obtain 200 sections and this has been accomplished for approximately 100 samples currently stored in a freezer.

Aim 2: This aim was designed to examine the impact of the loss of SELENOF on prostate pathology development in mouse models where SELENOF knockout mice are crossed with mouse models of prostate cancer. The first funding period saw little progress as there were challenges in obtaining the required mice. In the second funding period, breeding colonies of SELENOF knockout mice and HiMyc mice have been established and the appropriate genotypes established using PCR by both commercial testing and using our own designed primers.

To obtain the end genotype, mice that are both SELENOF knockout and transgenic for HiMyc requires two rounds of crosses. The first is a cross between SELENOF KO mice and the HyMyc mice which will yield offspring with one wild allele for SELENOF and Hi-Myc and a

backcross of these mice to yield SELENOF$^{-/-}$ and HiMyc mice which will be sacrificed at designated time points for examination. The first phase of breeding has been accomplished and once the pups are of age, they will be backcrossed for the generation of the experimental mice.

Aim 3: The goal of Aim 3 was to investigate the mechanism by which the loss of SELENOF contributes to prostate cancer progression. In the first funding period, the focus was on our initial success of knocking down SELENOF in RWPE-1 immortalized prostate cancer cells and the accomplishments were described in the annual report for that period. Our exhaustive efforts to over-express SELENOF in prostate cancer cells was unsuccessful, despite significant effort. The translation of SELENOF requires signal sequences in the 3'-untranslated regions (3'UTR) of the SELENOF mRNA that designates the single in-frame codon as the amino acid selenocysteine. Multiple constructs were tried with different parts of the 3'UTR as well as comparable 3'UTRs from other selenocysteine containing proteins. Finally, we tried to express SELENOF in a different cell type, the MCF-7 cell line derived from human breast carcinomas. As a first step, several breast cell lines were examined for SELENOF levels, including those that estrogen receptor (ER) positive, Her2 positive and derived from triple negative breast cancer (TN). MCF-7 cells were selected to test our SELENOF expression construct due to ease of culturing and low endogenous SELENOF levels (Figure 1).

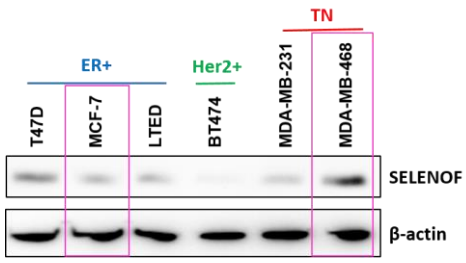

Figure 1. SELENOF levels in human breast cancer cell lines.

MCF-7 cells were stably transfected with one of our constructs that should express SELENOF from a doxycycline-inducible promoter. As shown in Figure 2A, exposure of transfected cells to doxycycline for 3 days resulted in robust induction of SELENOF. Having achieved enhanced SELENOF levels in these cells, proliferation assayed in several ways (Figure 2B-D).

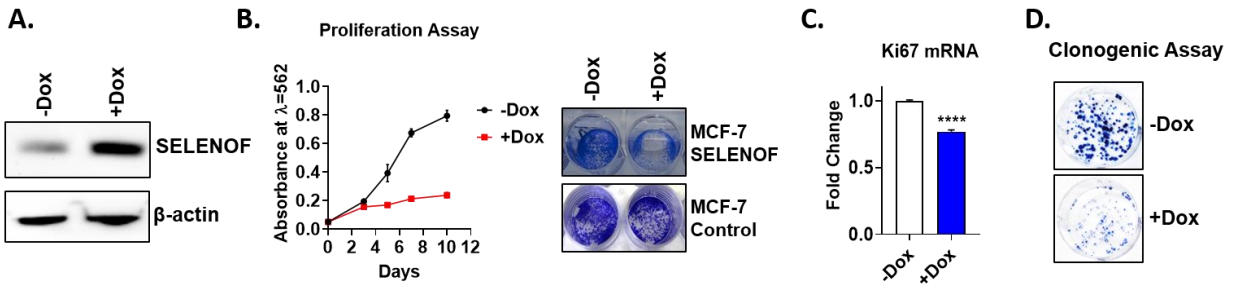

Figure 2. SELENOF overexpression in ER+ MCF-7 breast cancer cells attenuate aggressive phenotypes such as, proliferation and clonogenic survival. (A) Western blot for SELENOF levels in whole cell extracts of MCF-7 SELENOF cells treated without or with Dox (1µg/ml) for 72hrs. Stably transfected MCF-7 cells containing a Dox-inducible SELENOF expression construct (pRetroX-Tight-Pur-TetOn-Advanced). β-actin serves as a loading control. (B) Proliferation assay of MCF-7 SELENOF cells treated without or with Dox for 10 days. Cells are fixed and stained in crystal violet solution, then solubilized in 1% SDS. Data is quantified and shown as absorbance at 562nm (right panel). Representative pictures of stained wells are shown on the upper left panel. Control MCF-7 cells are shown on the bottom right panel. (C) The proliferation marker Ki67 is measured by RT-QPCR in cells treated as in Fig. 4A. (D) Clonogenic assays conducted in MCF-7 SELENOF cells treated twice weekly without or with Dox for 2 weeks. Dissociated single cells were seeded in 6 well plates at a clonogenic density of 1000 cells per well. Colonies were fixed and stained with crystal violet. Representative pictures are shown.

In order to continue to characterize cells that over-express SELENOF, additional assays were performed. A significant challenge to the management of breast cancer is the persistence of cells with stem-like properties, know to sustain tumor initiation and recurrence, and are shown to be resistant to standard cancer therapies. Mammosphere formation in undifferentiating and anchorage-independent conditions was used as a functional assay to assess the impact of SELENOF on the stem-like properties in MCF-7 cells. The induction of SELENOF with Dox resulted in a significant decline in the number of mammospheres compared to control cells (Figure 3A), indicating that SELENOF may suppress cancer stem-like cells and tumorigenesis. Tamoxifen (4-hydroxytamoxifen, 4OHT, is the active metabolite) is the archetype endocrine therapy against ER+ breast cancers.

Elevated SELENOF attenuates hormone (estrogen, E2)-induced proliferation, but more importantly, it enhances tamoxifen's anti-proliferative activity (Figure 3B). A similar effect was observed with fulvestant, another endocrine therapy drug (data not shown). Altogether, our data indicates that SELENOF contributes an important beneficial role by attenuating overall cell growth and clonogenic survival, reducing stem-like properties, and enhancing endocrine therapy response.

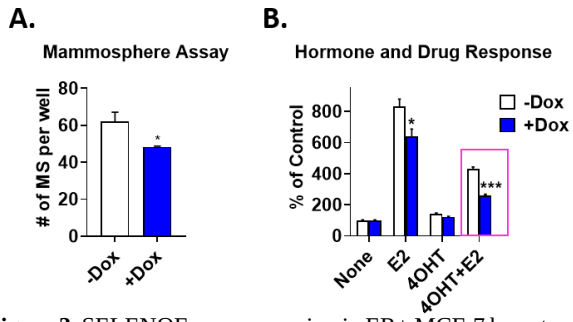

Figure 3. SELENOF overexpression in ER+ MCF-7 breast cancer cells attenuate mammospheres formation and enhances response to tamoxifen. (A) Mammosphere assay is conducted in MCF-7 SELENOF cells. Cells were seeded in low-attachment plates at single cell density. Methyl cellulose was added to prevent cell aggregations. After 7 days, mammospheres arising were measured and mammospheres >50µm in diameter are quantified. (B) MCF-7 SELENOF cells were treated with estradiol (E2, 10nM), 4-hydroxytamoxifen (4OHT, 1µM) or the combination for 5 days. Cells are fixed and stained in crystal violet solution, then solubilized in 1% SDS. Data is normalized to own controls. set to 100%.

In the report from the first funding period, we included data indicating that knocking down SELENOF in prostate cells resulted in a dramatic change in energy metabolism as measured by oxygen consumption, using the Seahorse platform. It was therefore examined whether over-expression of SELENOF in MCF-7 cells could attenuate oxygen consumption, using the same approach used on the prostate knock-down cells. As seen in Figure 4A, over-expression of SELENOF in MCF-7 resulted in the opposite effect we reported for the knock down cells, a reduction in oxygen consumption rate (OCR). Consistent with this observation is the change in the expression of several key enzymes involved in energy metabolism (Figure 4B).

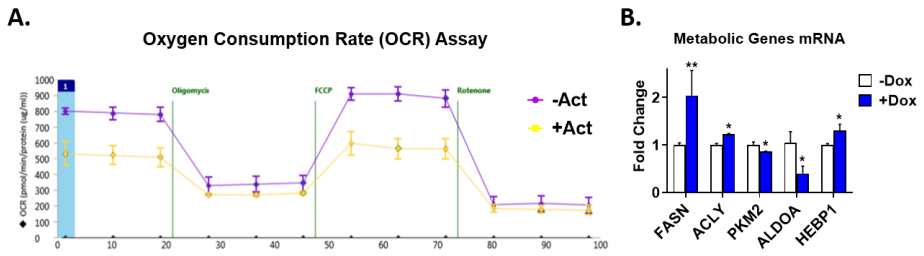

Figure 4. SELENOF impacts energy and metabolism. (A) Oxygen consumption rate (OCR) is measured using the Seahorse XF analyzer (Agilent Technologies, Inc). ACT is a tetracycline analog used to induce SELENOF expression in MCF-7 SELENOF cells. (B) Expression of selected metabolic genes measured by RT-QPCR in cells treated as in Fig. 4A as shown.

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

Dr. Elhodaky is an M.D. and was a Ph.D. student supported by the DOD award. He successfully defended his thesis in May and is currently be trained in residency in Pathology In addition to Dr. Elhodaky, an undergraduate student (Shrinidhi Kadkol) has participated in this project, becoming adept at molecular cloning and analysis, as well as receiving two monetary awards for his efforts on the project from UIC, an Honors Council Award and Liberal Arts and Sciences Undergraduate Research Initiative (LASURI). He graduated from the University of Illinois at Chicago in May and will continue his efforts on this project as a research technician during this upcoming year. He is already accepted to the medical school here at UIC for the fall of 2021 and hopes to be accepted to the MD/PhD program. He has submitted several abstracts for presentations at national meetings that were accepted, but unfortunately those conferences were cancelled due to the pandemic. Shrinidhi has written and submitted an invited manuscript that is currently under review and will soon start writing a research paper on which he will be first author. He is also scheduled to present his data to the department as part of our bimonthly "Works in Progress" seminar series, which will be presented to the Research Division within the Department of Pathology on Zoom.

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

Our research on SELENOF was included in a review published in Biological Trace Element Research with DOD support acknowledged as well as another article in "Nutrients" currently under review. Several abstracts for presentations at national meetings describing our progress on this project were accepted for presentations and Dr. Diamond was scheduled for an invited lecture at the University of Missouri, however all of these opportunities were cancelled due to the pandemic.

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

  Specific Aim 1: With the tumor bank becoming operational again, the rest of the clinical samples from the UIC tumor bank will be collected and the pathologist will review each sample and move forward on their processing for SELENOF localization and quantification, genotyping and selenium analysis.

  Specific Aim 2: Offspring from the SELENOF $^{-/+}$HyMyc backcross will be euthanized at intervals and prostatic tissue will examined by the pathologist to determine whether SELENOF loss accelerated prostate pathology as posed in our main hypothesis. With the resumption of research, we will also start the necessary breeding of the SELENOF knockout mice with mice PTEN mice to pursue studies addressing the second animal model of prostate cancer.

  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 resolve the mechanisms by which SELENOF ectopic expression is suppressed in prostate cell lines. To do so, we have generated a reporter construct in which the SELENOF 3-UTR is used to drive the translation of a luciferase reporter gene in which we introduced an in-frame UGA codon. Production of this mutagenized luciferase is directed by the SECIS element in the 3'-UTR and the efficiency of translation is quantifiable. This construct will be used to efficiently determine what translation factors are inhibiting SELENOF in prostate cells, and those factors will be suppressed. Resulting prostate cells will be examined for phenotypic and molecular changes, being guided to a large degree by the results described above in which SELENOF was successfully over-expressed in MCF-7 cells. The data obtained knocking down SELENOF in RWPE-1 prostate cells and the TMA data are now being compiled for publication.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**
  Our cell culture data has now clearly shown that altering the levels of SELENOF can impact features of the transformed phenotype, establishing that SELENOF loss is not a bystander in prostate cancer development. Mechanistically, we can now hypothesize that one means by which SELENOF can function as a tumor suppressor is by altering energy metabolism during cancer progression. By gaining insight as to the means by which SELENOF levels are reduced in cancer, it is now anticipated that novel therapeutics that can be designed to enhance SELENOF expression or compensate for its loss will be able to be designed.

- **What was the impact on other disciplines?**
  The data that SELENOF is reduced in prostate cancer and that it functions as a tumor suppressor, coupled with the data indicating that the African Americans express the SELENOF allele that is associated with increased risk of dying from this disease contribute to the broad issue of understanding the reasons behind the higher incidence and worse outcome of prostate cancer in African Americans. In addition, the results reported in breast cancer cells expands upon the original scope of the project and indicates that SELENOF loss may be relevant to another form of cancer in which health disparity in clinical outcome also exists.

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

- **What was the impact on society beyond science and technology?**
  There are broad impacts of this research on society's appreciation for the reasons of health disparities in cancer to innate features such as genotype. The results also bring to light the benefits of a healthy lifestyle, specifically diet, in impacting risk of disease.

5. Challenges/Problems

- **Changes in approach and reasons for change.**
  While all the proposed experiments were to be conducted in prostate cells, the difficulties with getting expression from the constructs in prostate cells resulted in testing the construct in breast cancer cell lines. Since the constructs were effective in enhancing SELENOF in MCF-7 cells, these results indicated that there was nothing fundamentally wrong with the construct itself. The transfected MCF-7 cells were therefore used to generate phenotypic and molecular data that will be used to selectively examine derivative prostate cell lines once the block to translation is resolved.

- **Actual or anticipated problems or delays and actions or plans to resolve them.**
  There were significant delays in the project due to the months of shut down that resulted from the pandemic. This not only affected those working on the project in my laboratory, but all the available support services including core services and the animal facility. Research in the College of Medicine at the University of Illinois at Chicago is now resuming under a strict set of restrictions. Animal breeding, sample collection and bench experiments were all suspended, but are now resuming.

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

  Expenditures associated with supplies and animal costs were much less due to the suspension of research due to the pandemic.

- **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.**

Selenoproteins of the Human Prostate: Unusual Properties and Role in Cancer Etiology
Diamond, A.M. Biol Trace Elem Res 192:51-59, 2019

The Interaction Between Dietary Selenium Intake and Genetics in Determining Cancer Risk and Outcome. Kadkol, S and Diamond, AM Nutrients, 2020 (under review)

  - **Books or other non-periodical, one-time publications.**
    Nothing to report

  - **Other publications, conference papers, and presentations.**

Abstracts were submitted and accepted on the work funded by this project and accepted for presentation at the national conferences of the American Association for Cancer Research and the American Society of Nutrition. Presentations were also scheduled in the Department of Chemistry at the University of Missouri and the "Selenium in Human Health" conference scheduled in Hawaii. Unfortunately, all these events were cancelled due to the pandemic.

  - **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. Maarten Bosland, qualified collaborator: No change
Dr. Mostafa Elhodaky, M.D., Ph.D.*
Michael Schlicht, technician: No change
Shrinidhi Kadkol, B.S*

Dr. Elhodaky has graduated with his Ph.D. and his effort has been replaced by Mr. Kadkol as a full-time technician, assuming Dr. Elhodaky's responsibilities on the project.

- **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 5% salary support from Dr. Donald Vander Griend's NIH project entitled "Function of the stem cell transcription factor SOX2 in prostatic enlargement.

  Dr. Balla is now receiving 3% effort on Dr. Donald Vander Griend's NIH project entitled "Function of the stem cell transcription factor SOX2 in prostatic enlargement and 3% effort on Dr. Nonn's DOD grant entitled "High Intraprostatic Androgens in African American Men Primes for Aggressive Prostate Cancer".

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

  Loyola University, Maywood, Illinois
  Dr. Irida Kastrati, has contributed to the project as a collaborator, providing advice and conducting some studies on breast cancer cells without compensation.

8. Special Reporting Requirements

- **Collaborative Awards:**
- **Quad Charts:**

9. Appendices