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TITLE: Epigenetic Modulation of SOS1 Gene Promotes Breast Cancer Disparity by Altering Immune Landscape

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13. SUPPLEMENTARY NOTES

In the US, more than 40,000 breast cancer patients succumb to this disease every year. The age-adjusted breast cancer incidence in AA women is lower compared to CA women. However, the mortality rates of AA women were 29.5 while the rates of CA women were 20.8. Thus, the cancer death rate in AA females is 14% higher than in CA female. However, exact reasons to explain this racial disparity is still yet poorly understood. The goals of the current proposal are to find genetic and non-genetic factors that influence the disease process that is specific to AA women so that we can identify the exact target to treat and prevent the aggressive breast cancer in this population. We hypothesize that AA women with obesity develop aggressive breast cancer by upregulation of the gene called *SOS1* gene through race/ethnic specific non-genetic control and also by tiny RNA called miR-483. We also hypothesize that our identified compound called taxifolin and its modified chemical (TF-10) selectively suppress the proposed pathway, thereby preventing lung metastasis of breast cancer. We will focus our next year's effort on further clarification of the SOS1 function on racial disparity.

15. SUBJECT TERMS
Breast cancer, Racial disparity, SOS1 gene

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1. Introduction

African American (AA) women are diagnosed with more aggressive breast cancers and have poorer survival than Caucasian (CA) women, despite that AA women have a lower incidence of the disease. The exact molecular mechanism of the racial disparity is still poorly understood. However, multiple genetic and epigenetic factors are believed to be involved. Critical questions are (i) what are the associated genetic factors and pathways?, (ii) why are these factors "activated" specifically in AA patients?, (iii) can we identify these risk factors in advance of disease?, and (iv) can we develop therapeutic and preventive drugs that target these factors? Based on these preliminary data, **we hypothesize** that AA women with obesity develop aggressive breast cancer by upregulation of the *SOS1* gene via race/ethnic specific epigenetic control of super enhancer and posttranscriptional modulation by miR-483. These changes result in (i) activation of PTTG1 signaling for cancer stem cell and resistance and (ii) activation of M2 macrophages with subsequent alteration of the immune landscape to promote metastasis. We also **hypothesize** that taxifolin selectively suppresses SOS1 function, thereby preventing tumor metastasis. To test these hypotheses, we propose (i) to elucidate the race/ethnic specific epigenetic control of the *SOS1* gene via super enhancer and miR-483 (**Aim 1**), (ii) to clarify the mechanism by which *SOS1* promotes metastatic progression through the activation of PTTG1 pathway and alteration of the immune landscape (**Aim 2**), and (iii) to test the selectivity and efficacy of taxifolin derivative in animal models (**Aim 3**).

2. Keywords

Racial disparity, Breast cancer, SOS gene, exosome, microRNA, taxifolin

3. Accomplishments

a. What were the major goals of the project

Aim 1: To elucidate the race/ethnic specific epigenetic control of the *SOS1* gene via super enhancer and miR-483.

(a) Determine whether SNPs in the super enhancer are responsible for SOS1 expression.
(b) Identify transcription factors involved in the super enhancer function.
(c) Clarify the mechanism by which miR-483 controls SOS1.

Aim 2: To clarify the mechanism by which SOS1 promotes metastatic progression through the activation of PTTG1 pathway and alteration of the immune landscape.

(a) Clarify how SOS1 activates the PTTG1 pathway.
(b) Elucidate the mechanism of how the SOS1/PTTG1 axis promotes cancer stem cells
(c) Examine how SOS1 alters the immune landscape through polarization of M2 macrophages.
(d) Examine how SOS1 promotes lung metastasis *in vivo*.
(e) Validate the shift of immune cell population with clinical samples.

Aim 3: To test the selectivity and efficacy of taxifolin derivative in animal models.

(a) Test the specific efficacy of taxifolin *in vitro* and *in vivo*.

(b) Test the efficacy of taxifolin and TF-10 in syngeneic xenografts and patient-derived xenograft (PDX) in humanized mice.

b. **What was accomplished under these goals?**

Aim 1: To elucidate the race/ethnic specific epigenetic control of the *SOS1* gene via super enhancer and miR-483.

We observe a strong enrichment of H3K27Ac signaling in the region encompassing ch2: 38662500–38672400 bases, which is located 500 kb downstream of the *SOS1* gene in AA cell lines, but not in CA cell lines, suggesting that these regions are highly activated and may affect the expression of neighboring genes, including *SOS1*. Interestingly, we found that this region includes an enhancer that spans approximately 1.3 kb (chr2: 38665459–38666743) defined by the Enhancer Atlas database. We also examined breast tumor samples from each race by ChIP-PCR analysis using anti-H3K27Ac antibody and found that this enhancer region is indeed significantly enriched in AA patients compared with CA patients. We, then deleted the super-enhancer region using the paired gRNA-mediated CRISPR technique designed by CRISPETa and validated the successful knockout of the sequence by PCR. As shown in **Fig. 1**, deletion of the enhancer indeed significantly reduced the SOS1 expression in three AA cell lines. Thus, our results identified a unique race-specific H3K27Ac-mediated gene regulation through the enhancer in AA patients, which promotes the SOS1 expression.

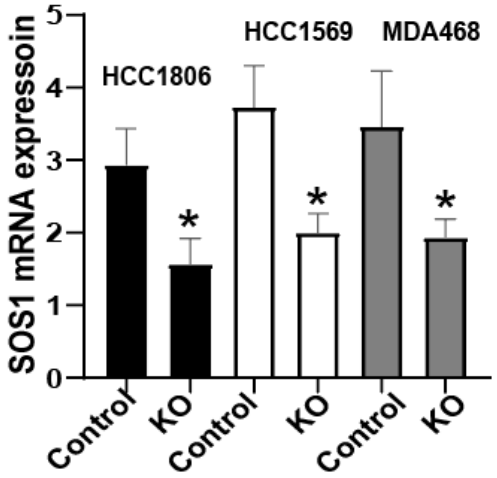

Figure 1. SOS1 is regulated by a super enhancer. Super enhancer was deleted using the paired-end sgRNA-mediated CRISPR technology in three AA cell lines, and the expression of SOS1 was examined by qRT-PCR. * $p<0.05$, ** $p<0.001$.

To examine whether histone modifications are involved in the downregulation of miR-483 in AA patients, we analyzed the ChIP-seq data derived from AA and CA cell lines. We found a strong enrichment of H3K27me3 signaling located upstream of the miR-483 gene promoter, which spans approximately 2 kb (**Fig. 2 left**, red boxed). Importantly, eight of 10 transcriptional start sites (red) that were predicted by miRStart website were located in this region, indicating a potential transcriptional suppression by histone trimethylation in the promoter of miR-483. To validate this hypothesis in clinical samples, we performed H3K27me3 ChIP-PCR in breast tumor tissues derived from AA and CA patients ($n = 4$ each) and found that this region is indeed highly tri-methylated in histone among AA samples compared with CA samples (**Fig. 2, right**). These results strongly suggest that miR-483 is downregulated in a racial-specific manner by

promoter histone trimethylation and that this downregulation results in upregulation of SOS1.

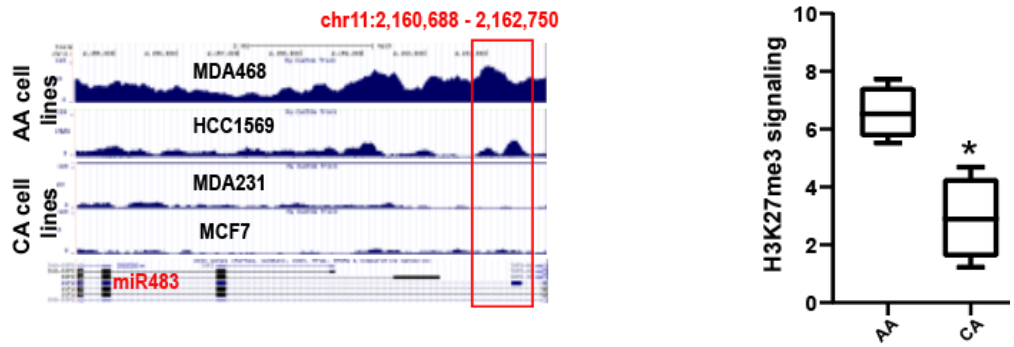

Figure 2. Down regulation of miR-483 promotes the SOS1 expression in AA patients. (Left) H3K27me3 signaling in the promoter (red boxed) of miR-483 of the indicated AA and CA cell lines. **(Right)** H3K27me3 ChIP-PCR analysis for the promoter region of miR-483 was done using clinical samples from AA (n=5) and CA (n=5) breast cancer patients. * p<0.05, ** p<0.001.

Aim 2: To clarify the mechanism by which SOS1 promotes metastatic progression through the activation of PTTG1 pathway and alteration of the immune landscape.

We attempted to identify key regulatory factors that are influenced by racial disparity using master regulator analysis, an unbiased bioinformatics approach that identifies key genes that are enriched in certain phenotypes from patients' genetic profiles. We found that PTTG1, which belongs to the c-Met signature gene, was the most significantly and differentially expressed regulator in both cohorts. We then examined the expression of PTTG1 in four AA cell lines with or without knockdown of the *SOS1* gene. The successful knockdown of SOS1 was accomplished by infecting cells with a mixture of short hairpin RNAs. As shown in **Fig. 3A** and **3B**, PTTG1 expression was significantly suppressed by knockdown of SOS1, suggesting that SOS1 plays a critical role in regulating PTTG1 expression. It has been reported that PTTG1 plays a critical role in the epithelial–mesenchymal transition (EMT) and self-renewal of cancer stem-like cells.

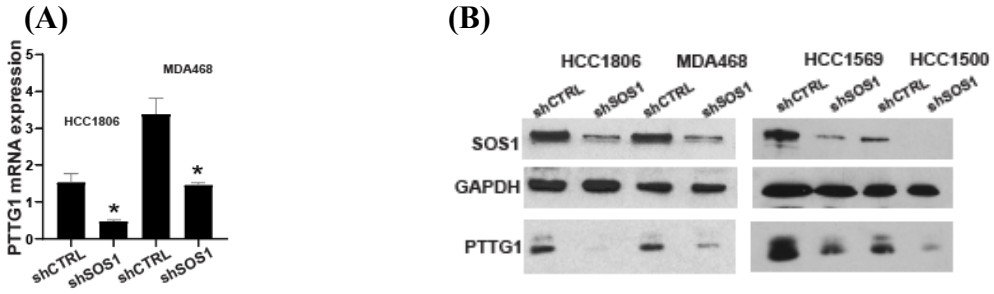

Figure 3. SOS1 mediated PTTG1 upregulation and enhanced stemness of cancer cells. (A) PTTG1 expression was examined in cancer cells with or without knock-down of SOS1 by qRT-PCR. **(B)** PTTG1 expression was examined in cancer cells with or without knock-down of SOS1 by Western Blot.

Aim 3: To test the selectivity and efficacy of taxifolin derivative in animal models.

The proposed in vivo experiments to test the efficacy of taxifolin are underway.

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

Dr. Kerui Wu has been supported by the current grant as a full time postdoctoral fellow. His appointment has been 12 months. He is a co-author for all three papers listed as item #6 below. He is the first author of Nature Communication paper.

Dr. Dan Zhao who is a postdoctoral fellow in our lab has been supported by this grant for his training. His appointment has been 5.75 months. He is also listed as co-author of the three manuscripts listed in item #6. He is now moved to the South Western University in Texas to do his second postoc training.

d. **How were the results disseminated to communities of interest?**
"Nothing to Report."

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

For Aim 1, we plan to (i) determine whether SNPs in the super enhancer are responsible for SOS1 expression, and (ii) identify transcription factors involved in the super enhancer function.

For Aim 2, we will execute the proposed experiments to (i) elucidate the mechanism of how the SOS1/PTTG1 axis promotes cancer stem cells, and (ii) examine how SOS1 alters the immune landscape through polarization of M2 macrophages.

For Aim 3, we continue our effort to test the specific efficacy of taxifolin *in vitro* and *in vivo*.

4. Impact

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

An estimated 266,000 new cases of invasive breast cancer will be diagnosed in the United States during 2019, and more than 41,000 breast cancer deaths are anticipated among women. African American (AA) women are diagnosed with more aggressive breast cancers and have poorer survival than Caucasian (CA) women. Four major disparities are evident between AA and CA patients: (i) early age of onset, (ii) more advanced stage of disease, (iii) more aggressive histologic changes, and (iv) shorter survival times. However, a comprehensive understanding of the basis for these disparities remains elusive. This project aims at finding novel genetic and epigenetic factors that affect tumor progression specifically to AA women in order to develop more effective and preventive approaches. The outcome of the proposed project will have direct impact on our understanding of breast cancer disparity, which will lead to a development of novel therapeutic and preventive measure. The proposal will have multiple impacts. **First**,

understanding the mechanism of SOS1-mediated disparity may lead to the development of preventive measures. **Second**, elucidating the roles of SNPs and super enhancer as well as miR-483 in the disparate expression of SOS1 may reveal a novel mechanism of breast cancer disparity. **Third**, understanding how SOS1 promotes M2 polarization and alters the immune landscape will provide a novel paradigm for disparity research. **Finally**, our discovery of taxifolin as a novel inhibitor for SOS1 activity may lead to development of a preventive approach for AA women at risk of breast cancer.

b. **What was the impact on other disciplines?**
"Nothing to Report."

c. **What was the impact on technology transfer?**
"Nothing to Report."

d. **What was the impact on society beyond science and technology?**
"Nothing to Report."

5. Changes/Problem

a. **Changes in approach and reasons for change**
None

b. **Actual or anticipated problems or delays and actions or plans to resolve them**
None

c. **Changes that had a significant impact on expenditures**
None

d. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
None

e. **Significant changes in use or care of human subjects**
None

f. **Significant changes in use or care of vertebrate animals.**
None

g. **Significant changes in use of biohazards and/or select agents**
None

6. Products

a. **Publications, conference papers, and presentations**

i. **Journal publications.**

1. Fei Xing1, Dan Zhao1, Shih-Ying Wu1, Abhishek Tyagi1, Kerui Wu1, Sambad Sharma1, Yin Liu1, Ravindra Deshpande1, Yuezhu Wang1, Jacob Cleary1, Lance Miller1, Amar Chittiboyina, Yin-Yuan Mo3, Kounosuke Watabe. Epigenetic and post-transcriptional modulation of SOS1 promotes breast cancer metastasis in African American women. (2021) **Cancer Research** 81(11):3008-3021.

2. Shih-Ying Wu, Sambad Sharma, Kerui Wu, Abhishek Tyagi, Dan Zhao, Ravindra Pramod Deshpande, and Kounosuke Watabe. Tamoxifen suppresses brain metastasis of estrogen receptor-deficient breast cancer by skewing microglia polarization and enhancing their immune functions. (2021) **Breast Cancer Research**, 23, 35-

3. Kerui Wu, Jiamei Feng, Fei Xing, Sambad Sharma, Yin Liu, Shih-Ying Wu, Dan Zhao, Abhishek Tyagi, Ravindra Pramod Deshpande, Xinhong Pei, Ravi Singh and Kounosuke Watabe Exosomal miR-19a and IBSP cooperate to induce osteolytic bone metastasis of estrogen receptor-positive breast cancer. **Nature Communications**, 2021. *12*(1). doi:10.1038/s41467-021-25473-y

ii. **Books or other non-periodical, one-time publications.**
None

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

1. Shih-Ying Wu, Sambad Sharma, Kerui Wu, Abhishek Tyagi, Dan Zhao, Ravindra Pramod Deshpande and Kounosuke Watabe. "Tamoxifen suppresses brain metastasis of estrogen receptor-deficient breast cancer by skewing microglia polarization and enhancing their anti-tumor immune functions" (2021): 2715-2715. Annual meeting of American Association for Cancer Research. Washington, D.C.
2. Abhishek Tyagi, Sambad Sharma, Kerui Wu, Shih-Ying Wu, Dan Zhao, Ravindra Deshpande, Kounosuke Watabe. "Nicotine promotes pre-metastatic niche formation in hormone receptor negative breast cancer through paracrine signaling in the tumor microenvironment". (2021)-A-1999-AACR, Annual meeting of American Association for Cancer Research, Washington DC.

b. **Website(s) or other Internet site(s)**
None

c. **Technologies or techniques**
None

d. **Inventions, patent applications, and/or licenses**
None

e. **Other Products**
None

7. Participants & Other Collaborating Organizations
What individuals have worked on the project?

Name:	*Ralph D'Agostino, Ph.D.*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	*Rdagostino (ecommons)*
Nearest person month worked:	*0.36*
Contribution to Project:	*Dr. D'Agostino* serves as a biostatistician for this project. He helped to design experiments and analyze the data for statistical validation.
Funding Support:	NIH (R01)

Name:	*Carl Langelfeld, Ph.D.*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	*Clangelfeld (NIH)*
Nearest person month worked:	*0.48*
Contribution to Project:	*Dr. Langelfeld* is an expert of human genetics and conducted SNP analysis for AA and CA patients.
Funding Support:	*NIH (R01)*

Name:	*Stacey O'Neill, MD,Ph.D.*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	*Soneill (ecommons)*
Nearest person month worked:	*0.6*
Contribution to Project:	*Dr. O'Neill* is a molecular pathologist and help collecting clinical samples and analyzing IHC data.
Funding Support:	*Pathology department fund*

Name:	*Fei Xing, Ph.D.*
Project Role:	*Co-I*
Researcher Identifier (e.g. ORCID ID):	*Fxing (ecommons)*
Nearest person month worked:	*0.6*
Contribution to Project:	*Dr. Xing* is a basic and translational researcher and he contributed the most in vivo experiment in this proposal.
Funding Support:	*NIH (R37)*

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

None

b. **What other organizations were involved as partners?**

None

8. Special Reporting Requirements

None

9. Appendices

None