AWARD NUMBER: W81XWH-17-1-0095

TITLE: Pax8: A Unifying Target for Serous Tumors

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CONTRACTING ORGANIZATION: University of Illinois at Chicago

REPORT DATE: August 2020

TYPE OF REPORT: Final Report

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2020	Final	4/15/2017 - 04/14/2020

4. TITLE AND SUBTITLE

Pax8: A Unifying Target for Serous Tumors

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0095

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
820 Chandler Street
Fort Detrick, MD 21702-5014

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

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

High-grade serous cancer (HGSC) may arise from the ovarian surface epithelium (OSE) or the fallopian tube epithelium (FTE). The paired-box transcription factor 8 (PAX8) is a transcription factor involved in the differentiation of Müllerian derived cells. The OSE does not express PAX8, but PAX8 is expressed in ~80- 96% of HGSC. Intriguingly, murine models of HGSC derived from the OSE acquire PAX8, suggesting that it is not only a marker of Müllerian origin, but also an essential part of cancer progression, potentially from both the OSE and FTE. Importantly, previous studies suggest that PAX8 expression is essential for the survival of HGSC regardless of source. Our preliminary data suggests that PAX8 loss in HGSC induces apoptosis, regulates migration, FOXM1, and angiogenesis. Targeting PAX8 may impact multiple aspects of ovarian cancer physiology and tumors derived from both OSE and FTE. Our preliminary data also indicates that reduction of PAX8 in normal oviductal cells does not significantly impact their survival, thus making it an interesting drug target. ***Our hypothesis is that PAX8 is an essential transcription factor for survival of HGSC regardless of cell of origin and blocking its expression may provide a new strategy for impacting both tumor cells and the microenvironment.***

15. SUBJECT TERMS

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
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION: High-grade serous cancer (HGSC) may arise from the ovarian surface epithelium (OSE) or the fallopian tube epithelium (FTE)(1–4). The paired-box transcription factor 8 (PAX8) is a transcription factor involved in the differentiation of Müllerian derived cells (5). The OSE does not express PAX8, but PAX8 is expressed in ~80-96% of HGSC. Intriguingly, murine models of HGSC derived from the OSE acquire PAX8, suggesting that it is not only a marker of Müllerian origin, but also an essential part of cancer progression, potentially from both the OSE and FTE (6–9). Importantly, our studies suggest that PAX8 expression is essential for the survival of HGSC regardless of source. Our data demonstrates that PAX8 loss in HGSC induces apoptosis, regulates migration, and FOXM1 expression (10). Targeting PAX8 may impact multiple aspects of ovarian cancer physiology and tumors derived from both OSE and FTE. Our data also indicates that reduction of PAX8 in normal oviductal cells does not significantly impact their survival, thus making it an interesting drug target (10). ***Our hypothesis is that PAX8 is an essential transcription factor for survival of HGSC regardless of cell of origin and blocking its expression may provide a new strategy for impacting both tumor cells and the microenvironment.***

BODY: We have made significant progress during year three of this no-cost extension for the pilot proposal. As outlined in our statement of work, our proposal had three aims. The first aim was completed and we published the majority of the data in our Oncogene paper in 2019.

Aim 2 of this proposal explored whether PAX8 contributes to tumor aggressiveness by regulating angiogenesis. As outlined in the previous progress report, we did not observe PAX8 affecting angiogenesis in our human tumor cell line. Hence, we do not plan to further pursue experiments outlined in Aim 2.

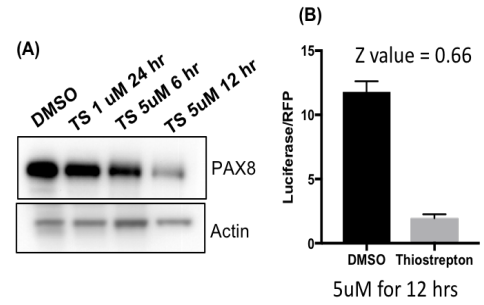

Figure 1. (A) Effect of thiostrepton on PAX8 expression in MOE cells (B) HTS assay developed and ready for screening

Our third aim was to develop a high-throughput screen (HTS) to find small molecules that can repress PAX8 promoter. To complete **Experiment 1A,** we have generated a stable MOE cell line expressing PAX8 promoter-luciferase. We have already engineered in a stable RFP virus driven by a CMV promoter as an internal control to counter screen for reduced cell viability. We have identified thiostrepton as a small molecule that decreases PAX8 protein levels (**Figure 1A**) and used it as a positive control for a preliminary screen assay. Using thiostrepton as a control (**Figure 1B**), the 'Z' factor for the assay is 0.6, which is an acceptable value for HTS. We optimized the assay for a 96-well format which was used to screen a small library of FDA-approved drugs (the Prestwick Library, ca. 1,000 members). In our previous progress report, we were able to validate that thiostrepton reduced PAX8, PKCalpha, FOXM1 expression, resulted in apoptosis, and was able to reduce PAX8 expression in high grade serous cancers *in vivo,* once encapsulated into micelles. However, because thiostrepton is insoluble, we wanted to have additional small molecules that we could use for future translation.

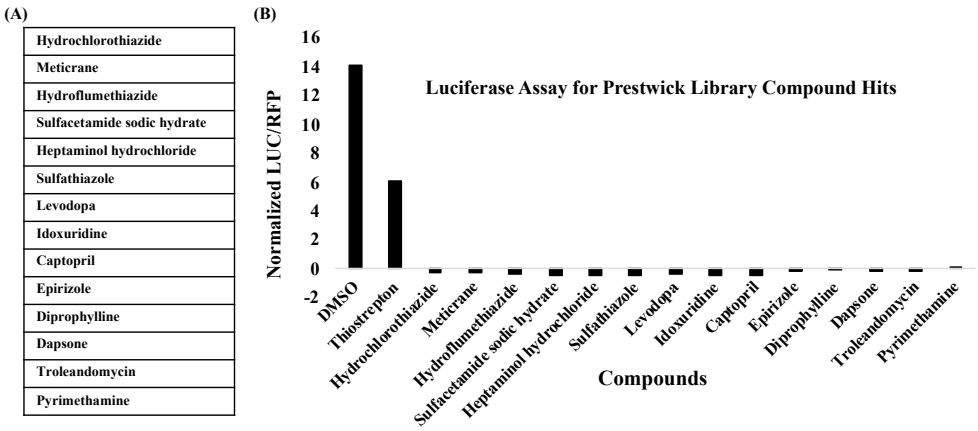

Figure 2. Fourteen compounds from the Prestwick library significantly reduced PAX8-luciferase expression.

From the Prestwick screen, we found another 14 compounds that were able to significantly reduce the PAX8-luciferase expression normalized to red fluorescent protein in our MOE reporter line (**Figure 2**). As a secondary screen to the luciferase reporter assay, we treated OVCAR8 cells with each compund and measured PAX8 protein by western blot (**Figure 3**). Thiostrepton (positive control), heptaminol hydrochloride, captopril, atracurium, and losartan were able to reduce PAX8 protein levels.

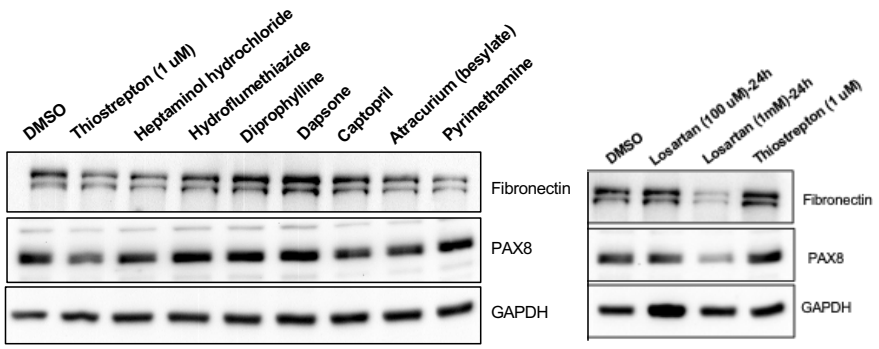

Figure 3: **OVCAR8 cells were treated with hits from the Prestwick library screening.** Cells were treated with 1 μM compound for 24hr before immunoblotting for PAX8 and fibronectin. GAPDH was used as a loading control.

Several papers have recently characterized PAX8 as a transcription factor(1-6), but very little has been published regarding the proteome or the secretome altered in HGSC when PAX8 is altered. Based on our own functional data demonstrating that PAX8 expression in OSE enhances migration and that loss of PAX8 in FTE and HGSC cells reduces this effect, we were interested in defining the secretome regulated by PAX8. RNA sequencing pathway analysis available from PAX8 silencing in multiple ovarian cancer cell lines all indicate a significant difference in transcripts that govern ECM and cytoskeletal rearrangements(*3, 6-8*). In order to investigate the secretome and confirm protein targets that are differentially expressed, we used mass spectrometry-based proteomics and with SILAC labeling of secreted proteins in serum free media. We found 749 differentially altered proteins and both PANTHER and GSEA analysis concluded that the major pathways regulated were TGFβ and ECM receptor interactions. Upon investigating the protein targets in these pathways, we hypothesized that PAX8 controls TGFβ1 expression, collagen 1 (COL1A1), and fibronectin. Interestingly, a major study recently found COL1A1 and fibronectin in the matrisome of high grade serous cancers as a major predictors of poor prognosis and reduced survival(*9*).

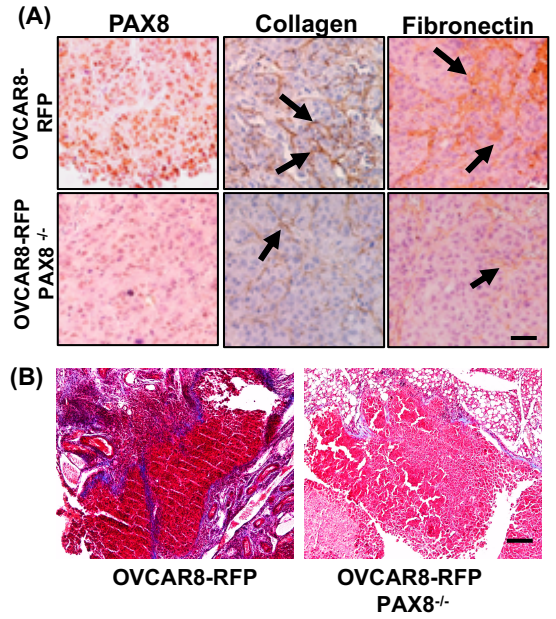

Figure 4. OVCAR8^{RFP}PAX8$^{-/-}$ have less collagen, fibronectin *in vivo* compared to OVCAR8^{RFP}. (A) IHC using PAX8, fibronectin and collagen 1 antibodies of tumor sections from xenografts. (B) Mason's Trichrome staining shows reduced collagen deposition in PAX8 deleted tumors.

Next, we took the OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ tumors that we had from the pilot award and stained them for collagen and fibronectin. In **Figure 4A**, the loss of PAX8 resulted in a dramatic downregulation of both collagen 1 and fibronectin staining in the tumor. Suboptimal cytoreductive associated networks, or SCAN, was previously demonstrated to be based to some extent on an increase in 'reactive stroma,' which is characterized by remodeling of ECM and the production of α-smooth muscle actin (α-SMA) and can be visualized with trichrome staining (*10*). We performed Masson's trichrome staining on the OVCAR8-RFP tumors with and without PAX8 and found significantly more collagen deposition in the PAX8 expressing tumors (**Figure 4B**).

We then generated another version of an HGSC model, the OVCAR4 cells. However, complete loss of PAX8 in OVCAR4 was lethal consistent with the essential role in HGSC survival, thus we used stable shRNA and clonal derivation to create a line of OVCAR4 that are knocked down (PAX8KD) for PAX8. Using both the OVCAR8 and the OVCAR4 models, cells were plated onto glass coverslips and immunostained for fibronectin. In both cases, the PAX8 reductions resulted in a dramatic loss of fibronectin (**Figure 5A**). In order to ensure that this also resulted in less secreted fibronectin, we took conditioned medium of the OVCAR8 cells and measured fibronectin in the media using western blot. Less fibronectin was seen in the conditioned medium from PAX8 deleted cells as compared to the parental line (**Figure 5C**). We then performed western on the whole cell lysate and found that both deletion or knockdown of PAX8 caused a decrease in the intracellular fibronectin (**Figure 5D**).

We have used this data as part of a TEAL EXPANSION application submitted this year. We hope with continued support, we will be able to focus on confirming that PAX8 is a regulator of ECM deposition in the tumor microenvironment through TGFβ production.

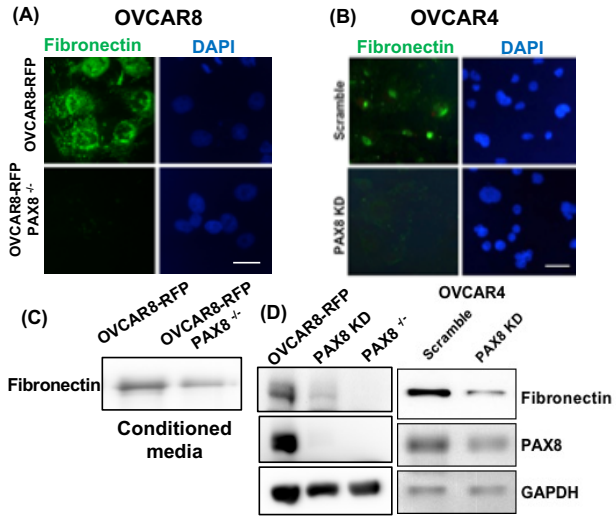

Figure 5. PAX8 reductions reduce fibronectin. Immunofluorescence for fibronectin and DAPI in OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ cells (A) or in OVCAR4 with PAX8 shRNA (B). (C) Immunoblot analysis for fibronectin using conditioned serum-free media from OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ cells. (D) Immunoblot analysis for fibronectin and PAX8 in OVCAR8^{RFP}, OVCAR8^{RFP} cells with PAX8 shRNA and PAX8 knockout or in OVCAR4. Actin and GAPDH are used as loading control.

KEY RESEARCH ACCOMPLISHMENTS:

- SILAC mass spectrometry analysis of OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ cells proteome and secretome. Validation of the secretome that collagen and fibronectin are regulated by PAX8.
- Generation of an MOE PAX8 promoter – luciferase cell line with an internal RFP control for a high throughput screen for compounds that inhibit PAX8 promoter activity. Identification of 14 compounds from the assay with an additional four validating in a secondary assay.
- *In vivo* study demonstrating targeting PAX8 using thiostrepton encapsulated micelles reduced tumor growth and PAX8 deletion reduces migration in human HGSC. This provides proof of concept for studies with our better lead small molecules.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- **Publications**

1. Hardy LR, Pelgardne MR, Esparza K, Lantvit DD, Heath K, Onyuksel H, Cologna SM, Burdette JE (2019) Proteomic analysis reveals a role for PAX8 in peritoneal colonization of high grade serous ovarian cancer that can be targeted with micelle encapsulated thiostrepton. Oncogene (in press).
2. Hardy LR, Salvi A, Burdette JE. (2018) UnPAXing the Divergent Roles of PAX2 and PAX8 in High-Grade Serous Ovarian Cancer. Cancers (Basel). 2018 Aug 8;10(8). pii: E262. doi: 10.3390/cancers10080262. Review.

- **Abstracts and presentations**

1. Hardy, L.R., Pergande, M.R., Esparza, K., Heath, K.N., Lantvit, D., Önyüksel, H., Cologna, S.M., Burdette, J.E. (2019) Proteomic analysis reveals a role for PAX8 in migration of high grade serous ovarian cancer. University of Illinois at Chicago College of Pharmacy Research Day (poster).
2. Hardy, L.R., Pergande, M.R., Esparza, K., Heath, K.N., Lantvit, D., Önyüksel, H., Cologna, S.M., Burdette, J.E. (2018) PAX8 increases migration and metastasis of ovarian cancer through upregulation of Rho GTPases. American Association for Cancer Research (AACR) Meeting (poster). University of Illinois at Chicago College of Pharmacy Research Day (poster).

- **Development of cell lines, tissue or serum repositories**

1) MOE cells expressing PAX8 promoter-luciferase construct with internal RFP control

Funding applied for based on work supported by this award

An NCI F30 training grant was awarded to the MSTP student Laura Hardy based on preliminary findings funded by this DOD award. She has since defended her PHD and returned to medical school. A Teal Expansion to support continued work was submitted in July 2020.

CONCLUSION: Our results demonstrate that PAX8 is a valuable drug target that reduces migration and metastasis in tumor cells derived from the OSE, FTE, and human HGSC. The OVCAR8^{RFP}PAX8$^{-/-}$ cell line had reduced tumor growth and increased survival compared to control OVCAR8^{RFP} cells. We have identified thiostrepton as a small molecule inhibitor that reduces the protein stability of PAX8. Mass spectrometry proteomics revealed that the protein interactome as well as the protein coverage of PAX8 is dramatically altered in response to thiostrepton. Thus, our studies show that targeting PAX8, either through CRISPR genomic alteration or through drug treatment with micelle encapsulated thiostrepton, leads to a reduction in tumor burden. Furthermore, we have developed a HTS with MOE PAX8 promoter-luciferase cells that uses thiostrepton as a positive control to inhibit PAX8 transcription. While loss of PAX8 triggers cell death limiting our ability to use CRISPR in most cell models except OVCAR8, we have been able to develop stable clones expressing shRNA targeted against PAX8 in murine oviductal tumor models (MOE cells), spontaneously transformed ovarian surface epithelium (STOSE), OVCAR3 and OVCAR4. Importantly, the technology will leverage our murine FVB/N tumor models to investigate the role of PAX8 and the immune system. From the pilot award, we have now identified two FDA approved molecules, both working in the Renin-Angiotensin pathway, that repress PAX8 protein levels. Both losartan and captopril are inhibitors of the renin-angiotensin pathway that block either active substrate production or the receptor. Our preliminary data found that losartan indeed reduces PAX8 protein levels and therefore its efficacy may be due to disrupting the stability of PAX8, which we found to be a master regulator of the tumor microenvironment.

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APPENDICES: N/A