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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.***

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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 two of this proposal. As outlined in our statement of work, our proposal had three aims. The first aim was to determine the incidence of HGSC when PAX8 is silenced in OSE and FTE derived serous models and in human HGSC cell lines. To complete **Experiment 1A**, we used CRISPR genomic editing to delete PAX8 from the human HGSC cell line OVCAR8. We performed a SILAC proteomics analysis of these cells in collaboration with Dr. Stephanie Cologna's laboratory. Several of the top differentially regulated pathways involved alterations to the cytoskeleton. Protein kinase Cα (PKCα) was one of the top upregulated proteins by PAX8 in OVCAR8, which has a known role in migration. We confirmed by immunoblotting that OVCAR8^{RFP} cells express higher levels of PKCα than OVCAR8^{RFP}PAX8$^{-/-}$ clones (**Figure 1A**). To investigate the functional significance of increased PKCα, we performed a wound closure assay for migration using the pan-PKC inhibitor GO6983. Inhibition of total PKC dramatically reduced migration of OVCAR8^{RFP} but this inhibition had no effect on migration of the OVCAR8^{RFP}PAX8$^{-/-}$ clones with already reduced PKCα levels (**Figure 1B**). Conversely, expression of the constitutively active PKCα (PKCαCAT) rescued migration in the OVCAR8^{RFP}PAX8$^{-/-}$ clones (**Figure 1C**).

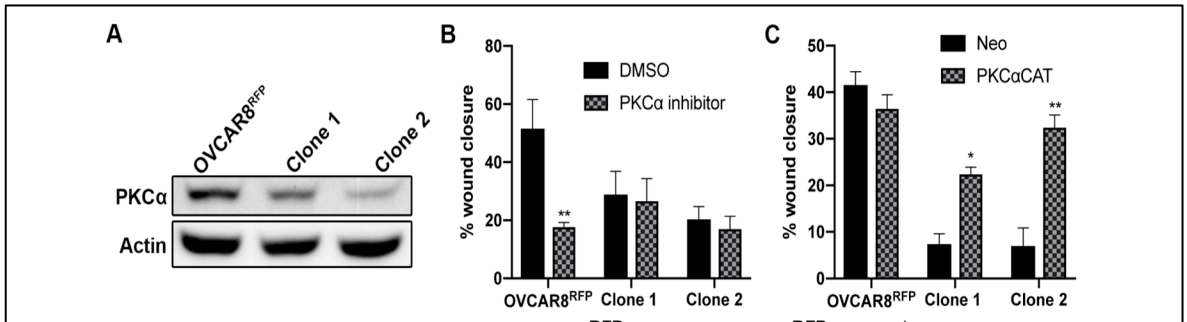

Figure 1. (A) Immunoblot for PKCα in OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ clones. (B) Wound closure assay performed with OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ clone 1 and clone 2 exposed to DMSO (control) or 5nM Go6976 (experimental) inhibitor against PKCα. (C) Wound closure assay (24 hours) with OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ clones transfected with neomycin vector (control) or PKCαCAT vector (experimental) containing the PKCα catalytic site.

We next performed an *in vivo* study with these cells by intraperitoneally (i.p.) injecting OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ cells into nude mice. Tumor metastasis was monitored using IVIS imaging system to detect RFP-tagged tumor cells. Mice injected i.p. with OVCAR8^{RFP}PAX8$^{-/-}$ cells developed tumors later than mice injected with wildtype OVCAR8^{RFP} cells and had increased survival (**Figure 2A**). Notably, over half the mice injected with OVCAR8^{RFP}PAX8$^{-/-}$ Clone 2 survived until the end of the study. Histological analysis confirmed Clones 1 and 2 had no detectable PAX8 protein within the tumor cells. PAX8 deletion also reduced PKCα levels *in vivo*, validating our previous *in vitro* findings (**Figure 2B**). These data indicate PAX8 is an important component of HGSC that drives tumor progression and aggressiveness via regulation of PKCα.

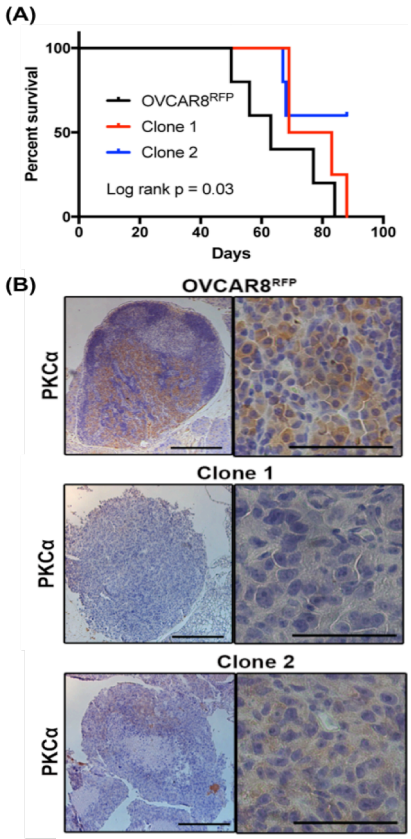

Figure 2. (A) Kaplan Meier survival curve. (B) Histological staining for PKCα in tumor cells.

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.

Our third aim was to develop a high-throughput screen (HTS) to find small molecules that can repress PAX8 promoter. To complete **Experiment 3A,** 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 3A**) and used it as a positive control for a preliminary screen assay. Using thiostrepton as a control (**Figure 3B**), the 'Z' factor for the assay is 0.6, which is an acceptable value for HTS. Currently, we are optimizing the assay for a 96-well format which will be used to screen a small library of FDA-approved drugs (the Prestwick Library, ca. 1,000 members). Once we have identified promising compounds that reduce PAX8 transcription, will perform dose- and time-course experiments and screen the hits for their ability to block PAX8 mRNA and protein in the validated HGSC cell lines OVCAR4, OVCAR8, and Kuramochi. We will monitor whether PAX8 reduction precedes cell death. and confirm that small molecules that block PAX8 also reduce downstream pathways such as FOXM1

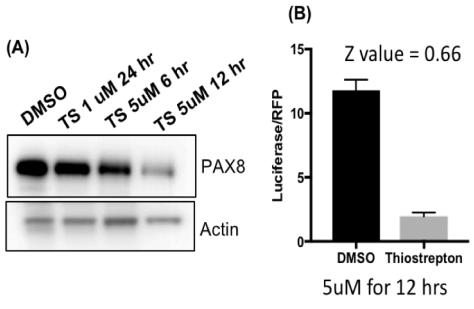

Figure 3. (A) Effect of thiostrepton on PAX8 expression in MOE cells (B) HTS assay developed and ready for screening

and PKCα.

Since thiostrepton decreases PAX8 protein levels, it could be used therapeutically to inhibit both PAX8 and FOXM1, two important regulators of HGSC aggressiveness. We i.p. xenografted OVCAR8-RFP cells and i.p. administered thiostrepton (250mg/kg/week) to the mice. However, this attempt was not successful due to the poor solubility of thiostrepton subsequently leading to toxicity issues. Hence, we encapsulated thiostrepton in DSPE-PEG$_{2000}$ micelles (TST-SSM) (**Figure 4A**) which improved the drug's aqueous solubility and allowed safe administration to animals. As shown in **Figure 4B**, TST-SSM significantly reduced the tumor burden *in vivo* compared to empty micelles (SSM). Thus, an enhanced therapeutic response to thiostrepton was achieved via this delivery method. Tumors treated with thiostrepton micelles showed reduction in PKCα and PAX8 expression by immunohistochemistry as shown in **Figure 4C**.

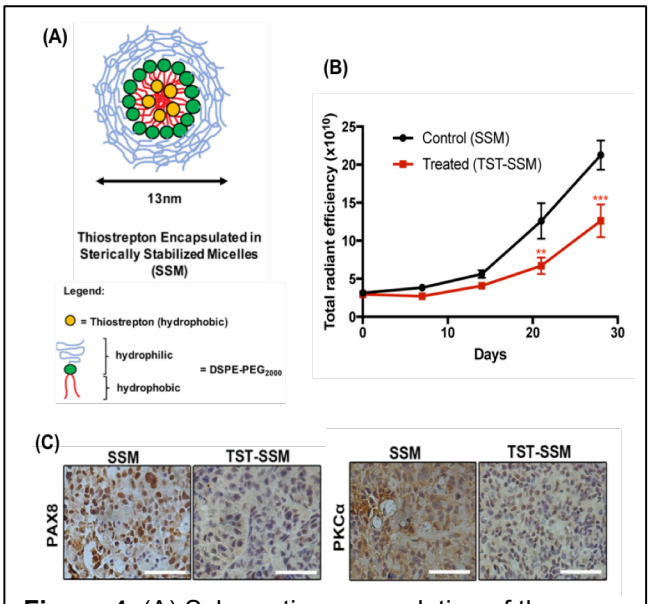

Figure 4. (A) Schematic encapsulation of the hydrophobic thiostrepton drug in sterically stabilized micelles (SSM) composed of distearoylphosphatidylethanolamine (DSPE-PEG) with a PEG chain of 2000. (B) Quantification of the total radiant efficiency obtained by IVIS imaging demonstrates reduced tumor burden in TST-SSM treated mice. (C) IHC demonstrates reduction in PAX8 and PKCα in tumors treated with TST-SSM.

Thiostrepton caused reduction of PAX8 protein levels via post translational modifications (PTMs) (Oncogene *in press*). Our preliminary data suggests the presence of PAX8 SUMOylation in ovarian cancer cells.

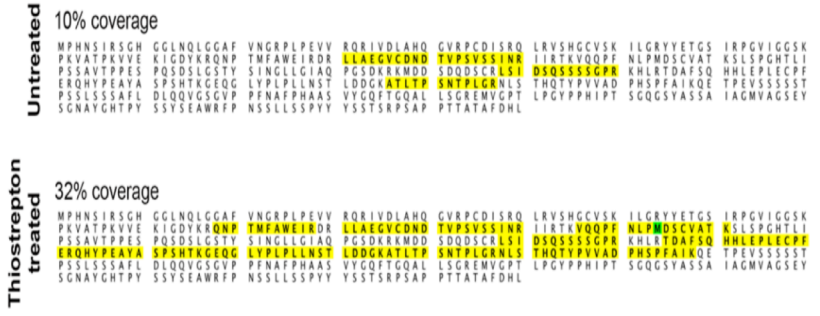

Figure 5. Protein coverage of PAX8 with and without thiostrepton reveals increased coverage after drug treatment

Immunoprecipitation of the PAX8 protein in the HGSC cell line OVCAR8, followed by detection using an SDS-PAGE gel, indicated the PAX8 protein to be approximately 70kDa. The expected size of unmodified PAX8 is 48kDa and addition of a SUMO body increases protein size by approximately 20kDa on an SDS-PAGE gel. Only 10% of the PAX8 protein was detected after trypsin digest in untreated cells but increased to 32% after thiostrepton treatment (**Figure 5**). An increase in protein coverage with thiostrepton suggests these modifications are lost after thiostrepton treatment. Literature supports that in thyroid cells, PAX8 is stabilized after a single deposition of SUMOylation at lysine residue 309 (11). Interestingly, the K309 residue in PAX8 with the predicted SUMOylation site was detected only after thiostrepton treatment.

Further, since we see major differences in the proteins bound to PAX8 with and without thiostrepton treatment, we predict that these interactions may play a role in protein stability. We will determine if this modification impacts protein stability in FTE and HGSC and we will test if this occurs due to the same K309 SUMOylation. Currently, we are performing immunoprecipitation and site-directed mutagenesis studies to confirm whether PAX8 is SUMOylated at K309.

KEY RESEARCH ACCOMPLISHMENTS:

- SILAC mass spectrometry analysis of OVCAR8 and OVCAR8^{RFP}PAX8$^{-/-}$ cells proteome and secretome.
- PAX8 increases migration of HGSC tumor cells by regulating PKCα.
- 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.
- *In vivo* study demonstrating targeting PAX8 using thiostrepton encapsulated micelles reduced tumor growth deletion reduces migration in human HGSC.

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

- **Publications**

1. Hardy LR, Pelgande 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. An R01 to support continued work was submitted in February 2019.

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. This HTS assay can uncover new chemical scaffolds that impact PAX8 expression either at the transcriptional level and also potentially at the translational level. Altogether, this work increases our understanding of the role of PAX8 in OSE and FTE derived cancer and highlights the promising potential for a high throughput screen that identifies compounds that decrease PAX8.

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APPENDICES: N/A