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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). 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.***

BODY: We have made significant progress during year one 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 confirmed by immunoblotting that our OVCAR8^{RFP}PAX8$^{-/-}$ clones had decreased PAX8 and FOXM1 at the protein level (Figure 1A). PAX8 deletion also reduced expression of the epithelial marker N-cadherin while increasing expression of the mesenchymal marker E-cadherin (Figure 1B). These findings suggest PAX8 regulates EMT in HGSC. Initially, we proposed to also delete PAX8 from the HGSC cell lines OVCAR4. While we were able to create a heterozygous OVCAR4 PAX8$^{+/-}$ clone with PAX8 knockdown and decreased FOXM1 levels, the homozygous deletion was lethal. We therefore continued our experiments with the OVCAR8^{RFP}PAX8$^{-/-}$ clones. 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 (Table 1). To functionally confirm if these cytoskeleton alterations affect cellular migration, we performed a wound closure and xCELLigence assay. OVCAR8^{RFP}PAX8$^{-/-}$ clones had decreased migration compared to control and rescue of the PAX8 deletion using a CMV-PAX8 promoter reversed the migratory defect (Figure 1B). A Boyden Chamber assay demonstrated PAX8 deletion also decreased the invasive abilities of ovarian cancer cells. We next performed an *in vivo* study with these cells by intraperitoneally (i.p.) injecting OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ cells into nude

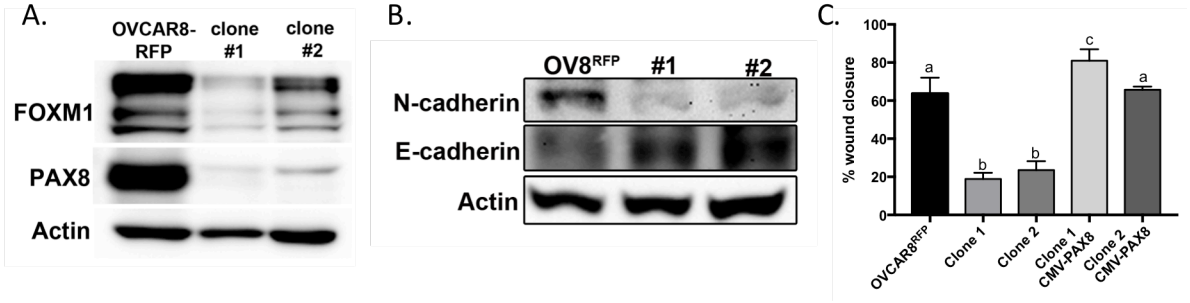

Figure 1. (A) Immunoblot for PAX8 and FOXM1 in OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ clones. (B) Immunoblot for EMT markers N-cadherin and E-cadherin. (C) Quantification of a wound closure assay for migration.

mice. 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,B). 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 (Figure 2C). Proliferation rate, which was quantified as the number of positive Ki67 puncti, demonstrated that tumors containing PAX8 proliferated faster than tumors with PAX8 deleted. These data indicate PAX8 is an important component of HGSC that drives tumor progression and aggressiveness.

Table 1. Top differentially regulated pathways after PAX8 deletion in OVCAR8 identified by mass spectrometry.

Gene ontology pathway	P value	Hits in pathway
Cytoskeleton structure	0.0005	11
Microtubule binding	0.001	15
Chaperone binding	0.001	9
Cytoskeletal protein binding	0.001	7
Ubiquitin protein ligase binding	0.007	16
Protein kinase binding	0.018	18
Cadherin binding involved in cell-cell adhesion	0.0038	14

As outlined in **Experiment 1B**, to determine if PAX8 differentially impacts tumor formation in OSE and FTE derived models of HGSC, we used siRNA to knockdown PAX8 in spontaneously transformed OSE (STOSE) and murine oviductal epithelium (MOE) PTEN^{shRNA}/KRAS^{G12V} cell lines (Figure 2A). These tumor models are derived from the murine OSE and FTE, respectively. In the original proposal, we suggested using a MOSE cell line that lacked PTEN and LKB1. This cell line, however, has relatively weak expression of PAX8 while the STOSE cell line has significantly higher expression of PAX8. We therefore continued our project using the STOSE cell line. PAX8 knockdown in both STOSE and MOE PTEN^{shRNA}/KRAS^{G12V} led to decreased migration. These results highlight the importance of PAX8 on migration in tumor models derived from both the OSE and FTE. Our continued characterization of these cell lines will include immunoblotting for FOXM1 and EMT markers such as N-cadherin, Fibronectin, and SLUG that we have previously shown to be regulated by PAX8. The results from Aim 1 are currently being prepared in a manuscript for publication.

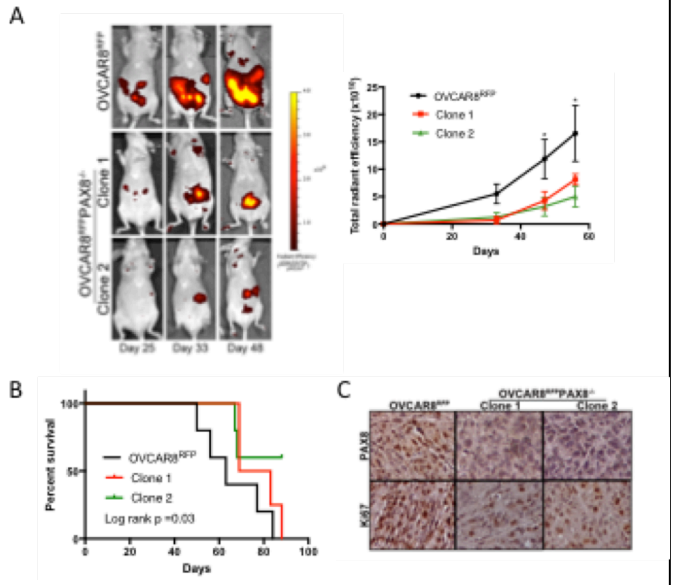

Figure 2. (A) IVIS imaging of RFP tagged tumor cells in mice injected i.p. with OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ cells. Quantification of total radiant efficiency measured using IVIS. (B) Kaplan Meier survival curve. (C) Histological staining for PAX8 and Ki67 in tumor cells.

Aim 2 of this proposal explored whether PAX8 contributes to tumor aggressiveness by regulating angiogenesis. As outlined in **Experiment 2A**, we examined the effect of PAX8 on angiogenesis in our OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ clones. SILAC labeling and mass spectrometry analysis of the secretome after PAX8 deletion did not identify alterations in secreted angiogenic factors. Using the *in vivo* study described in Aim 1 of this proposal, there were no significant changes in ascites production after PAX8 deletion. We will next measure

tumor vessel density in our tumor sections. However, based on our preliminary findings, it seems PAX8 does not affect angiogenesis in human HGSC. This was surprising to us because our MOSE-PAX8 RNA sequencing data identified over 40 pro-angiogenic mRNA transcripts upregulated upon PAX8 overexpression. We hypothesize that OVCAR8, which is a highly metastatic cell line, may have additional genetic alterations that increase their angiogenic potential. Since we did not observe PAX8 affecting angiogenesis in our human tumor cell line, we do not plan to further pursue experiments outlined in Aim 2.

Our third aim was to develop a high-throughput screen for molecules that inhibit PAX8. To complete **Experiment 3A,** we have generated a MOE cell line expressing PAX8 promoter-luciferase. EG1 is a small molecule that has been shown to disrupt PAX8 transcriptional activation and we have identified thiostrepton as a small molecule that decreases PAX8 protein levels. In our hands, thiostrepton reduced PAX8 transcription while EG1 did not (Figure 3). Going forward, we will use thiostrepton as a positive control for the high-throughput screen. Once we have identified promising compounds that reduce PAX8 transcription, we will perform a secondary screen to determine if these compounds reduce PAX8 in the HGSC cell lines OVCAR4, OVCAR8, and Kuramochi.

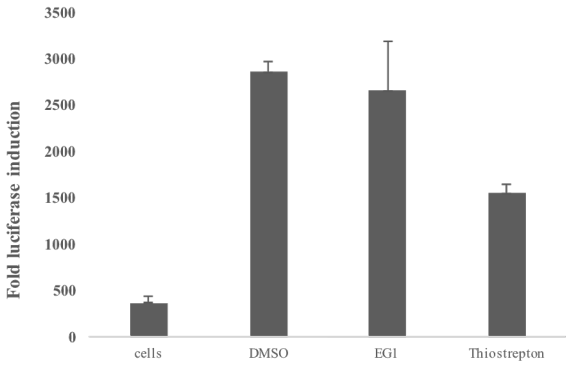

Figure 3. Luciferase assay in MOE cells with PAX8 promoter-luciferase exposed to DMSO, EG1, or thiostrepton.

We have previously shown that thiostrepton decreases PAX8 protein levels. Addition of thiostrepton to MOSE cells with a constitutively active viral CMV-PAX8 promoter still resulted in decreased PAX8 (Figure 4A). This suggests thiostrepton is affecting PAX8 protein stability rather than affecting transcription of PAX8. We also combined thiostrepton with the proteasome inhibitor MG132. Addition of MG132 did not rescue the effect of thiostrepton on PAX8, therefore indicating that thiostrepton is not stabilizing the proteasome to decrease PAX8 (Figure 4B). Our next step will be to determine if thiostrepton alters post-translational modifications on PAX8 that may lead to its degradation.

OVCAR8 cells exposed to thiostrepton have increased cleaved-PARP, an indicator of apoptosis (Figure 4C). We hypothesize that this increase in apoptosis is due to reduction in PAX8 and FOXM1. To test this, we will next expose OVCAR8^{RFP}PAX8$^{-/-}$ cells to thiostrepton. We hypothesize that there will be less apoptosis in this cell line, demonstrating thiostrepton induces apoptosis by reducing PAX8.

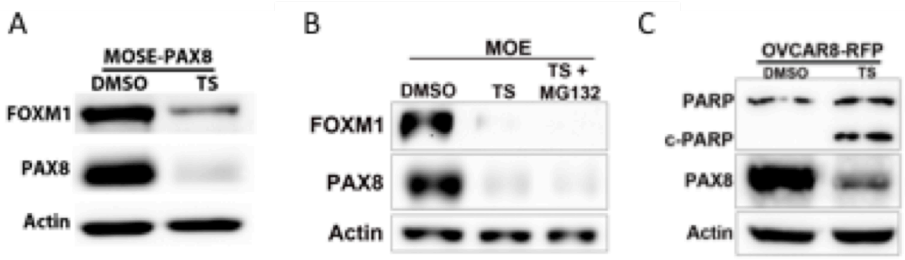

Figure 4. (A) Immunoblot for PAX8 in MOSE CMV-PAX8 cells exposed to thiostrepton. (B) Immunoblot for PAX8 in MOE cells exposed to the proteasome inhibitor MG132 and thiostrepton. (C) Immunoblot for cleaved PARP in OVCAR8 cells exposed to thiostrepton.

KEY RESEARCH ACCOMPLISHMENTS:

- Generation of two CRISPR genomically edited OVCAR8 cell lines with PAX8 deleted.
- SILAC mass spectrometry analysis of OVCAR8 and OVCAR8^{RFP}PAX8$^{-/-}$ cells proteome and secretome.
- *In vivo* study demonstrating PAX8 deletion reduces migration in human HGSC.
- PAX8 knockdown in tumor models derived from the OSE and FTE reduces migration.
- Generation of a MOE PAX8 promoter – luciferase cell line for a high throughput screen for compounds that inhibit PAX8 promoter activity.
- Identification of thiostrepton as a small molecule inhibitor of PAX8 protein stability that leads to tumor cell death.

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

- **Abstracts and presentations**

Hardy, L.R., Pergande, M.R, Cologna, S.M., Burdette, J.E. PAX8 increases migration and metastasis of ovarian cancer through upregulation of Rho GTPases. American Association for Cancer Research (AACR). (Poster)

Hardy, L.R., Pergande, M.R, Cologna, S.M, Burdette, J.E. Proteomic and transcriptomic analysis of the ovarian surface epithelium with PAX8 overexpression identifies a novel regulatory role for PAX8 in the focal adhesion pathway. Society for the Study of Reproduction (SSR). (Poster)

Pergande, M.R., Hardy, L.R., Bhat, V., Haney-Ball, C., Burdette, J.E., Cologna, S.M. Multi-omics anlaysis for the validation of differential transcripts in PAX8 overexpressed MOSE cells. US HUPO 2017. (Poster)

Pergande, M.R., Hardy, L.R., Bhat, V., Haney-Ball, C., Burdette, J.E., Cologna, S.M. A Study of Label-Free and Isobaric Tag Approaches for the Detection of Biologically Relevant Changes in PAX8 Overexpressed MOSE cells. American Society for Mass Spectrometry (ASMS). (Poster).

Hardy, L.R., Burdette, J.E. Transcription factor PAX8 protein is destabilized by the natural product thiostrepton. American Physician Scientist Association Annual Meeting (APSA). (Poster).

Hardy, L.R., Burdette, J.E. Transcription factor PAX8 protein is destabilized by the natural product thiostrepton. Chicago Cancer Biology Retreat. (Poster).

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

The CRISPR genomically edited OVCAR8^{RFP}PAX8$^{-/-}$ cell line was developed for this research proposal.

- **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.

CONCLUSION: Our results demonstrate that PAX8 increases migration in OSE and FTE derived serous models and in the human high grade serous cell line OVCAR8. The OVCAR8^{RFP}PAX8$^{-/-}$ cell line had reduced tumor growth and increased survival compared to control OVCAR8^{RFP} cells. Ascites production, however, was unchanged *in vivo* and our secretome data did not identify alterations in pro-angiogenic proteins between OVCAR8^{RFP} and OVCAR8^{RFP}PAX8$^{-/-}$ cells. These findings taken together suggests PAX8 is a valuable drug target that reduces migration and metastasis in tumor cells derived from the OSE, FTE, and human HGSC but may not drive angiogenesis *in vivo*. We have identified thiostrepton as a small molecule inhibitor that reduces the protein stability of PAX8. This effect on protein stability is independent of the cell's proteasome activity. We have developed a high throughput screen with MOE PAX8 promoter-luciferase cells that uses thiostrepton as a positive control to inhibit PAX8 transcription. PAX8 is known to activate its own promoter, therefore allowing us to use thiostrepton as a transcriptional inhibitor. 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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4. Di Palma, T. *et al*. A role for PAX8 in the tumorigenic phenotype of ovarian cancer cells. *BMC Cancer* **14**, 277-287 (2014).
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APPENDICES: N/A