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14. ABSTRACT

Many now believe that the fallopian tube epithelium is the progenitor cell type for high-grade serous ovarian carcinoma. The initial phase of tubal cell metastasis could involve several sets of molecules, each of which represents a possible therapeutic target for intervention that would block serous cancer while still confined to the fallopian tubes. Using a series of normal, modified, and tumorigenic tubal cell lines, we will investigate the properties that allow tubal cells to migrate and adhere to novel three-dimensional ovarian organ cultures. We will also determine if the ovarian factors are necessary to fully transform fallopian tube cells. Using these as experimental models of pathway-modified or tumorigenic cells of tubal origin, we will investigate if ovarian factors enhance migration as a mechanism to explain the presence of tumor mass in the ovary of serous patients. 3D ovarian organ culture conditioned medium will be used as the chemoattractant. Collagen is a well-established matrix utilized by serous cancer cells, of unknown origin, to seed metastatic sites, such as the mesothelium. An RNAseq analysis will be performed between human TEC adhered to collagen matrix compared to tissue culture plastic and used to identify gene expression changes responsible for adhesion on collagen. Ovarian conditioned medium (OCM) with and without H_2O_2 treatment will be added to normal and our series of pathway-modified oviductal cell lines to determine if proliferation and growth in soft agar are enhanced by factors in the OCM.

15. SUBJECT TERMS

Nothing listed

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Table of Contents

INTRODUCTION: The goal of this grant proposal is to understand how the ovary contributes to the migration, adhesion, and transformation of serous tumors that are derived from the fallopian tube. The initial phase of tubal cell metastasis could involve several sets of molecules, each of which represents a possible therapeutic target for intervention that would block serous cancer while still confined to the fallopian tubes. Using a series of normal, mutated, and tumorigenic tubal cell lines, we have investigated the properties that allow tubal cells to migrate to novel three-dimensional ovarian organ cultures, adhere to 3D collagen surfaces, and respond by proliferating to secreted ovarian factors.

BODY: We have made significant progress and achieved the major deliverables for year 1 of our proposed research. As outlined in our statement of work, our proposal had three aims. The first aim was to determine if secreted factors enhance migration of tubal epithelial cells. Our preliminary data had identified IP 10 (CXCL10), TIMP1, KC, MCS-F, and CCL2 (also known as MCP1). In addition, a paper was published suggesting that stem-like cells isolated from a high

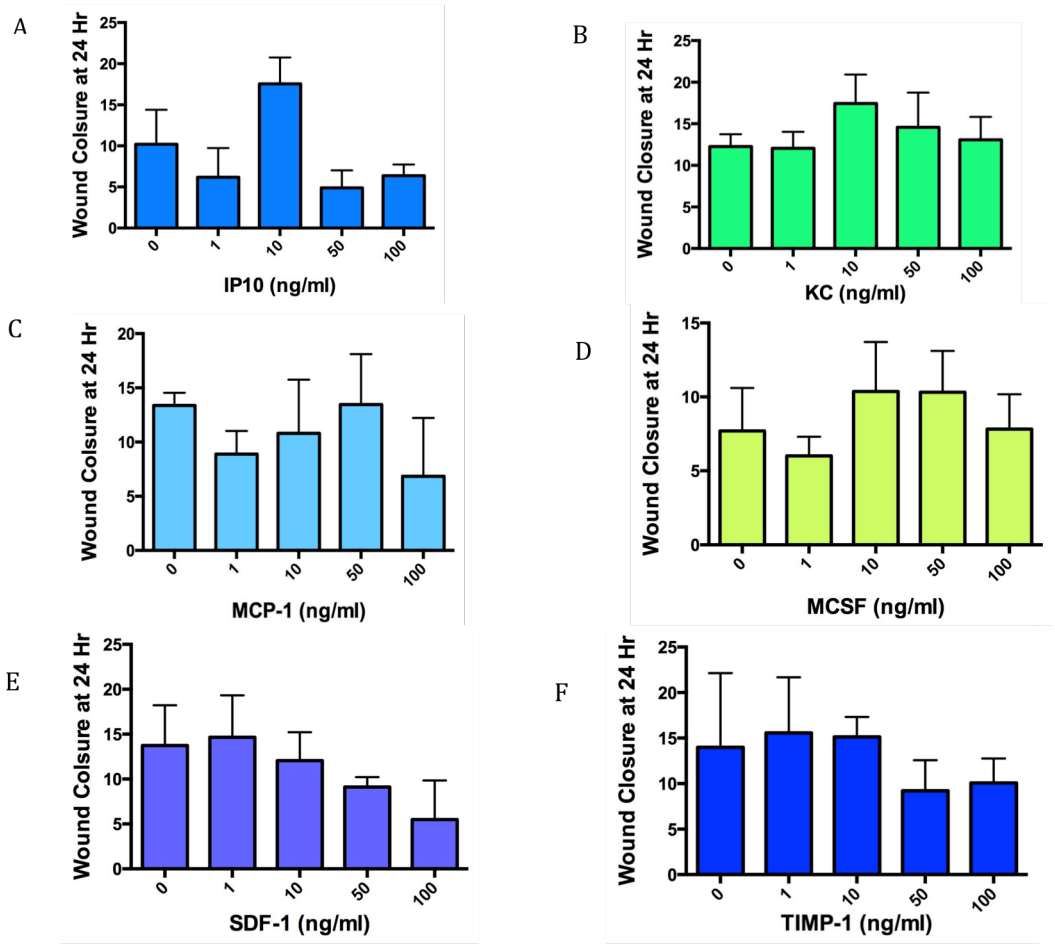


Figure 1. **Effects of Cytokines on Cell Migration. (A)** MOE cells were treated with different concentrations (0, 1, 10, 50, and 100 ng/ml) of cytokines and an analysis of wound closure was done using scratch migration assay. No significant change in wound closure was observed between cells in the treatment groups and cells in the control group. n=3.

grade serous cancer patient of unknown cellular origin was attracted to the ovary because of SDF-1[1]. These were all tested on the normal murine oviductal cells (MOE) and none of them significantly increased migration (Figure 1).

Since this result was a surprise, and not what we anticipated, we next re-examined the conditioned medium and confirmed that it enhanced fallopian tube migration (Figure 2). We also compared ovarian conditioned medium to medium conditioned with 3D oviductal cultures to control for factors secreted by a tissue which tumors are not predicted to be driven to colonize. While oviductal conditioned medium increased migration, ovarian conditioned medium increased it to a much greater extent. Next, we exposed a panel of modified cell lines to understand how common players in serous tumors may modify fallopian tube cells to migrate to the ovary. We found that mutations in p53 (R273H and R248W) and mutation in KRAS (G12V) actually reduced migration in response to conditioned medium (Figure 3A and 3B),. Deletion of PTEN enhanced migration of the fallopian tube cells in response to conditioned medium (Figure 3D) and the combination of PTEN loss with mutation of p53 retained the PTEN phenotype of enhanced migration (Figure 3E). Lastly, the fallopian tube cells that has PTEN loss and mutation of KRAS were the most stimulated to migrate in response to ovarian conditioned medium. These results are consistent with the amount of peritoneal colonization that we found when these models were xenografted, where the PTEN/KRAS model was the most aggressive, followed by PTEN alone and then PTEN/mutantp53[2].

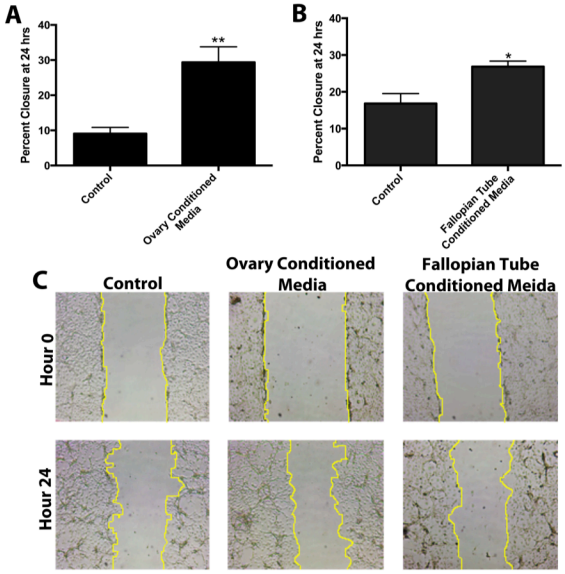


Figure 2. Ovarian conditioned medium drives fallopian cell migration.

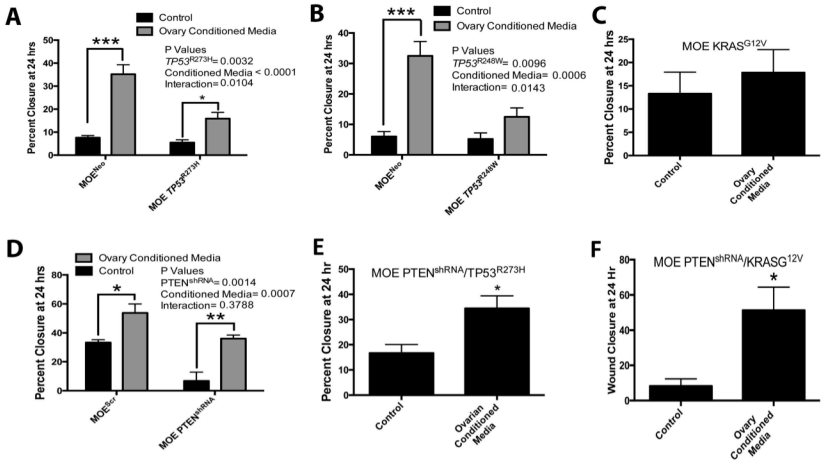


Figure 3. Key pathways in serous tumors modify migration to the ovary.

Since we now did not have any likely targets, we set out to determine if the secreted factor that enhanced cell migration was a protein or a small molecule. First, we ran the conditioned

medium through a molecular weigh cut-off filter and found that the stimulatory action was in the larger than 3 kDa molecular weight size (Figure 4A), indicative of a protein. Next we heated the solution, which denatures proteins and should result in a reduction of biological action if the active constituent is a protein. We found that heated conditioned medium no longer enhanced fallopian tube migration (Figure 4B). Finally, we subjected the conditioned medium to proteinase K and then inhibited the action with co-incubation with alpha2-macroglobulin and were able to demonstrate that enzymatically degrading proteins destroys the pro-migratory action (Figure 4C). Collectively these data indicate the active component(s) of the conditioned media is a protein.

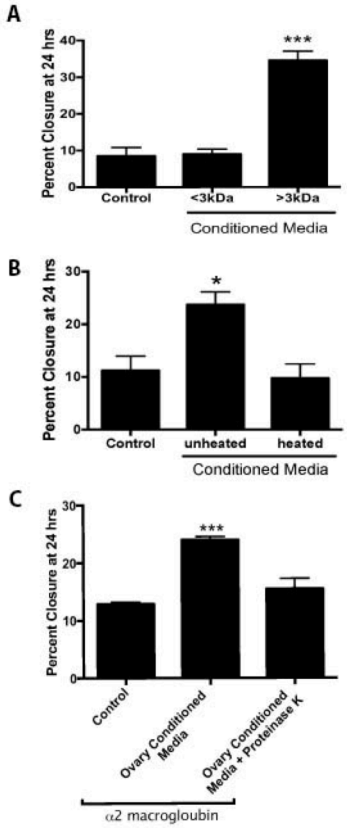
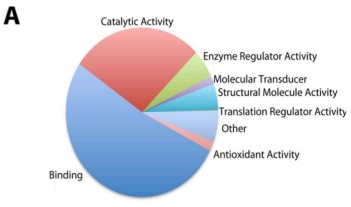


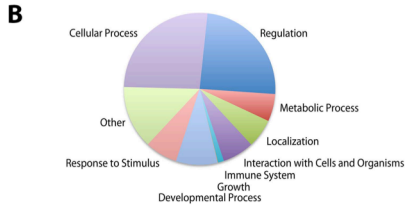
Figure 4. Pro-migratory factor in conditioned medium is a protein.

In order to characterize the proteins in the media, we evaluated the higher than 3kDa molecular weight fraction by mass spectrometry. We found more than 600 proteins in the conditioned medium. We then used bioinformatics databases to classify the action of these proteins. First we used molecular function in the program STRAP (Figure 5A) and DAVID (the Table in 5A). This revealed that many of the proteins are involved in binding and catalytic activity. We then followed this up using biological processes in STRAP (Figure 5B) and David (the Table in 5B) and found that these are cellular process and cellular regulation.

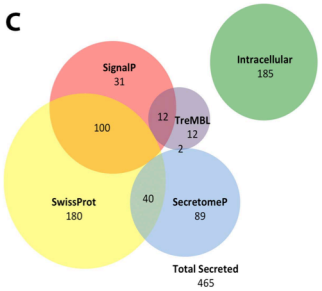
Because some of the proteins could be found in the media that are not secreted but rather represent cellular contents from dead cells, we used several databases to narrow the list into predicted proteins that are secreted. We hypothesized the secreted proteins are the ones most important to signal from the ovary to the fallopian tube that enhance migration. Using Uniprot we identified about 320 proteins are in the curated database (Swissprot- yellow circle Figure 5C) and some of



Term	Count	Percent	P-value
Catalytic Activity	235	46.1	$8.3x10^{-13}$
Antioxidant Activity	13	2.5	$1.8x^{-9}$
Structural Molecule Activity	35	6.9	$1.8x10^{-6}$
Binding	384	75.3	0.002
Enzyme Regulator Activity	33	6.5	0.028



Term	Count	Percent	P-value
Cellular Process	362	71.0	$8.8x10^{-15}$
Metabolic Process	282	55.3	$1.2x10^{-9}$
Cellular Component Biogenesis	35	6.9	$9.7x10^{-4}$
Cellular Component Organization	81	15.9	0.0026
Immune System Process	35	6.9	0.037
Response to Stimulus	89	17.5	0.072



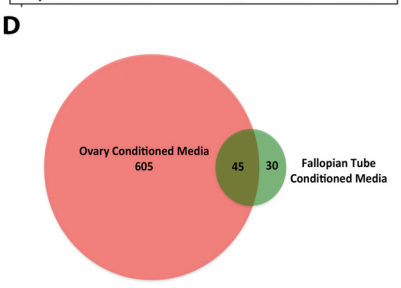


Figure 5. Mass spectrometry based proteomics and bioinformatics mining of secreted proteins from the ovary.

these have classical secreted signals as catalogued in the SignalP data set (Red circle/overlapping in the dark orange). In addition, Uniprto also has non-curated entries (TreMBL) and some of those overlapped with the SignalP data indicating that they are likely secreted proteins. The database SecretomeP uses six non-classical epitopes to find secreted proteins that lack a consensus secretion signal, and therefore the Secretome P and SignalP data are mutually exclusive. Overall we found that most of the proteins in conditioned media (465) are likely to be actively secreted and a minority of the proteins (185) likely present in the media due to cell death. While this validates the potential of our system to identify novel ovarian secreted proteins, we still had a large list (Figure 5C).

In order to try to eliminate some suspects, we analyzed the oviductal conditioned medium. We hypothesized that if there are overlapping subjects, those are not of interest because they are not ovarian specific factors that would drive migration. However, the oviduct had far fewer secreted proteins, and only 45 overlapped with the ovary (Figure 5D). Therefore, we still have a large list. We have begun a series of size exclusion chromatography experiments to further purify the active proteins. We have already determined that the most active proteins are between 50-100kDa and are using mass spectrometry based proteomics and additional separation steps to find the biologically active protein. In addition, we are using chemical inhibitors of key receptors that govern migration and are expressed in serous tumors to narrow our focus. Despite this being an alternative direction for our proposal, this analysis will actually give us a much better idea of how the ovary is influencing fallopian tube migration because we are utilize non-biased approaches instead of just looking at candidates. Also, it helps us keep the system more biologically relevant because the proteins are in the concentration that would be found naturally being secreted by the ovary. Since we did not want to mix murine protein samples onto human cells, once we have identified the key drivers, we will complete the experiments to stimulate the human ovarian cancer cell lines.

For specific Aim 2, our focus was to monitor how cells adhere to 3D collagen. First, we developed a protocol to isolate RNA from cells grown on 3D collagen, using TRIzol to lysis the cells followed by isolation of the RNA using silica-membrane RNeasy spin columns. Next, we optimized the length of time fallopian tube cells need to cultured on 3D collagen by monitoring expression of CTGF via qPCR, which was previously shown to be downregulated in response to 3D collagen in SKOV3 and DOV13 ovarian cancer cells. Fallopian tube cells grown on 3D collagen demonstrated a significant decrease in CTGF expression after six hours (Figure 6A) and also demonstrate a change in morphology consistent with altered adhesion.

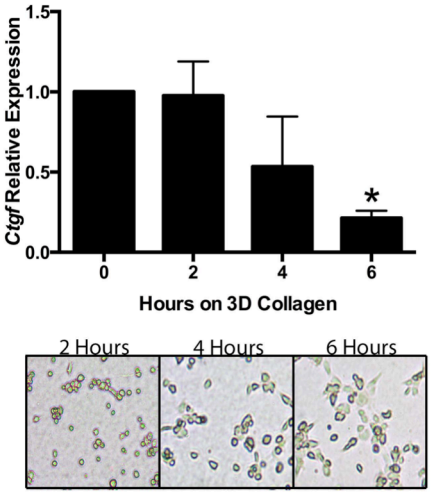


Figure 6. Fallopian tube cells on collagen demonstrate decreased CTGF after six hours.

Next we took several human high grade serous cancer cell lines and compared their morphology when grown on 2D and 3D collagen. We hypothesized that since previous data modeled

metastasis of cancer cells onto 3D collagen surfaces as a model of adhesion to the omentum, that this was late stage metastasis. Instead, exposed surfaces in the ovary, which are also rich in collagen, would be colonized by fallopian tube cells and that this response would differ between normal fallopian tube cells and malignant models. We found that the fallopian tube cells actually flattened out onto collagen more robustly than human high grade serous cancer cell lines (Figure 7). Intriguingly, the viability of the human high grade serous cancer cell lines on the 3D collagen was significantly better than the fallopian tube cell lines. These experiments allowed us to optimize the growth conditions for performing RNAseq on the fallopian tube cells and compare the regulated transcripts to the mRNA from previous results, obtained from our collaborator Dr. Barbolina, on DOV13 on 3D collagen.

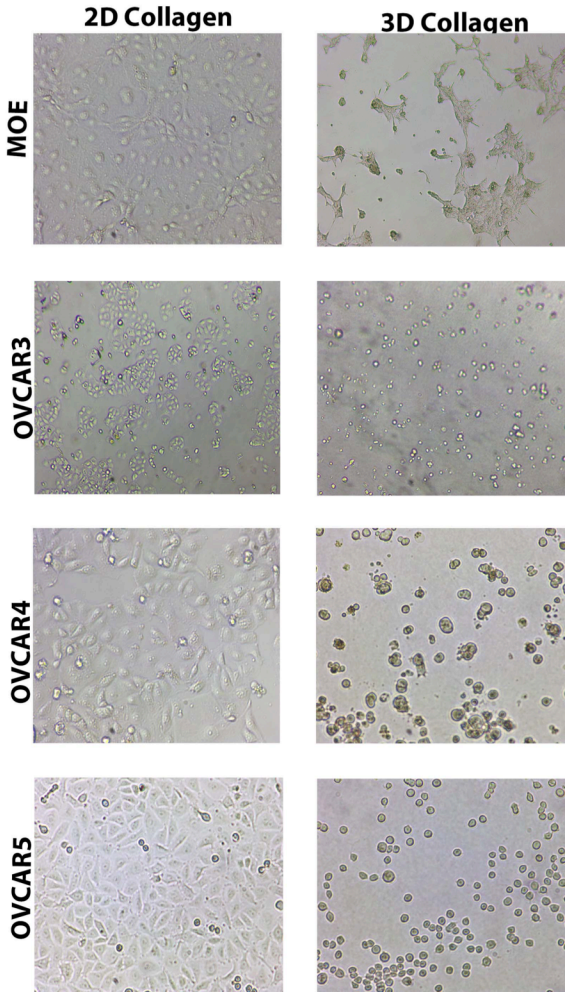


Figure 7. Morphology of fallopian tube cells and serous lines on 3D collagen.

As outlined in our proposal, we performed RNAseq on the fallopian tube cells grown on 3D collagen as compared to 2D. The results were verified to show repression of CTGF similar to the qPCR. The results are shown in Figure 9. The volcano plot in Figure 9A shows the most highly up and downregulated genes in terms of fold change and p value. When KEGG pathway analysis was completed, the most highly downregulated genes were involved in cellular adhesion and cytoskeletal rearrangement, which correlates with the biological process of colonizing a 3D collagen surface. The most highly upregulated pathways were the MAPK signaling pathway, though intriguing most of the unregulated genes in MAPK signaling actually inhibit MAPK. Our next step is to block with shRNA and/or small molecules the top hits from the RNA seq and monitor how this impacts 3D collagen adhesion. Further, we have also mined our data and compared it to the DOV13 microarray published by our collaborator. While they

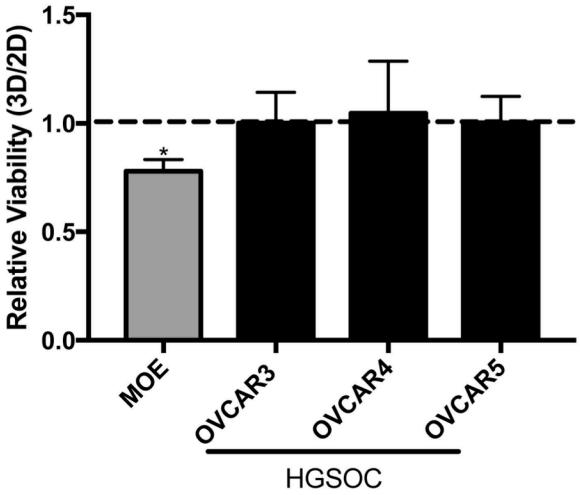


Figure 8. Relative growth on 2D vs 3D of fallopian tube cells compared to high grade serous.

found changes in WNT signaling, in particular DKK1, we also found WNT changes, but in WNT7A. We are actively pursuing changes in WNT as part of fallopian tube movement and adhesion to the ovary. We are also monitoring how our murine models with changes in PTEN, KRAS, and mutant p53 interact with the 3D collagen and how this changes signaling in WNT, adhesion, and MAPK. All of these signals could be blocked to help prevent initial ovarian metastasis.

For Aim 3 in the proposal, we planned to focus on how H2O2 and ovarian conditioned medium impacted fallopian tube proliferation and transformation. First, we pursued the impact of hydrogen peroxide on fallopian tube cells. Our previous papers illustrated that the doses we selected damaged DNA, but did not drive proliferation of the fallopian tube cells, even though this did increase proliferation and growth in soft agar of ovarian surface cells grown in the 3D ovary culture embedded in alginate. We also employed two additional mutagens to try and transform the fallopian tube cells, but all of them resulted in cell death without enhancing proliferation or growth in soft agar. Previously, we used continuous passaging to generate a spontaneously transformed fallopian tube cell line (We called it MOE High). This line formed tumors in mice and had an upregulation of the FOXM1 pathway. However, when we treated this tumorigenic line with H2O2, it did not have any differential response between the tumor forming and the normal cells, indicating that this strategy did not work.

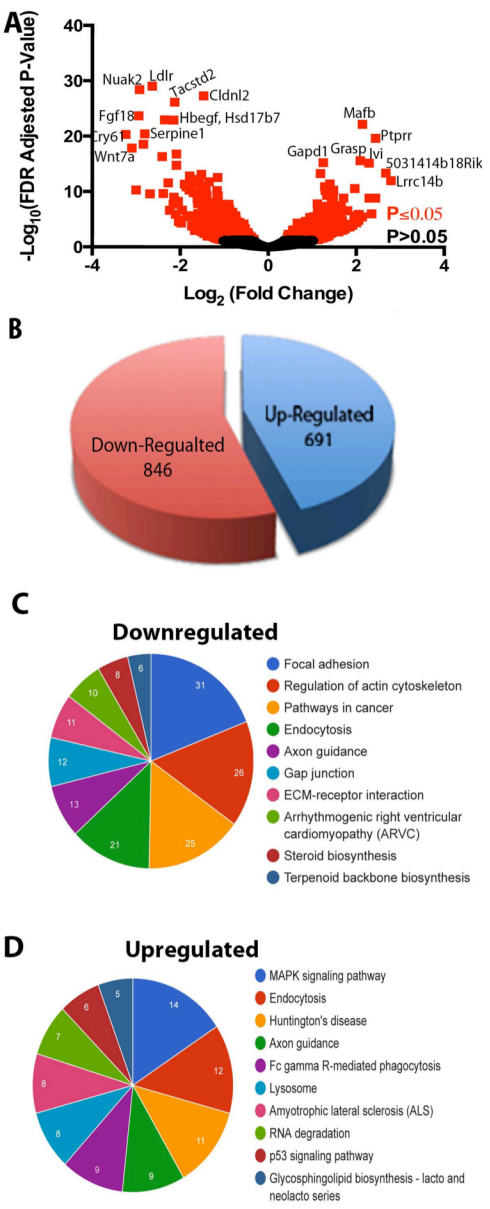


Figure 9. RNA sequencing volcano plot showing most high regulated transcripts on 3D compared to 2D collagen. KEGG pathway analysis shows the biological signaling pathways most highly regulated up and down.

Next, we subjected the fallopian tube cells to conditioned medium of the ovary and the oviduct. We wanted to include the control of the oviductal conditioned medium in case the effect was not specific to the ovary, but instead was true of any reproductive tissue. In fact, we found that both oviductal conditioned medium and ovarian conditioned medium enhanced the proliferation rate of the fallopian tube cells grown in culture. Therefore, we will follow-up on the alternative direction of stimulating the human high grade serous lines with conditioned medium from the murine ovarian organ culture and hope to identify through our efforts in Aim 1 any factors that cross react between the species that influence proliferation of the oviductal epithelium.

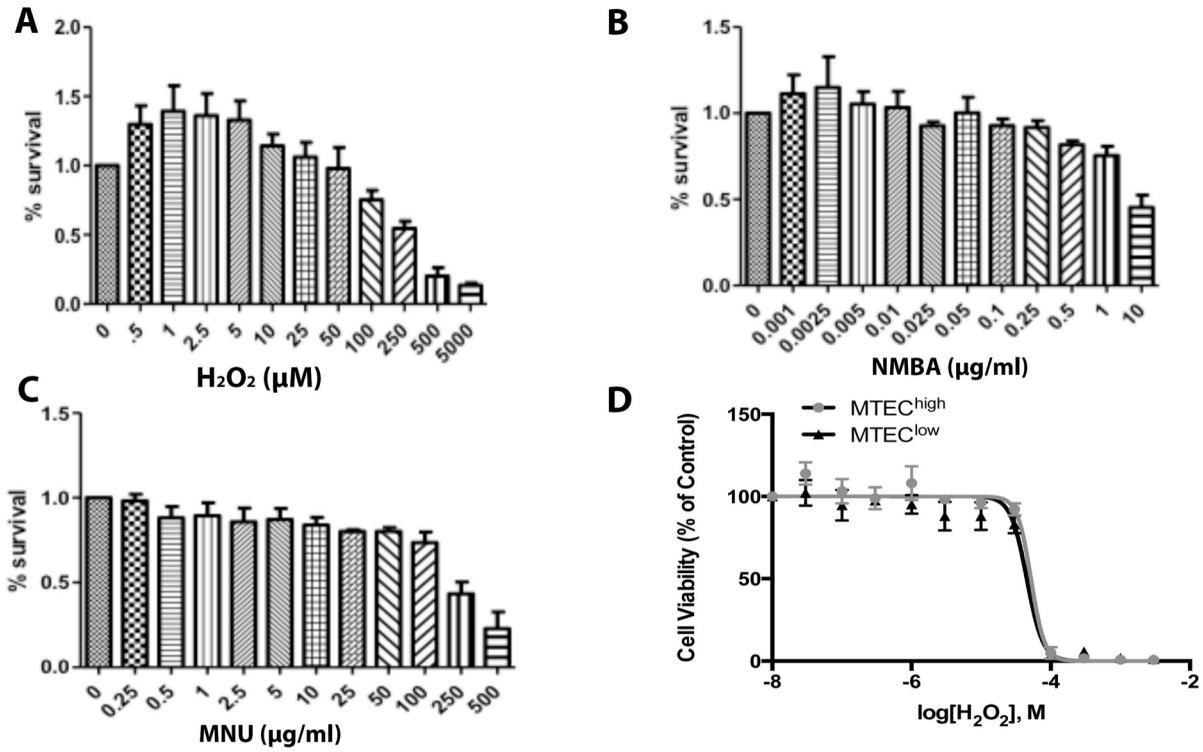


Figure 10. Fallopian tube cells exposed to various chemical mutagens did not survive and were not growth stimulated (A-C). Spontaneously tumorigenic fallopian tube cell lines derived from continuous passaging (called MOE high) was not differentially stimulated to proliferate in response to oxidative stress as compared to normal cell control (MOE low).

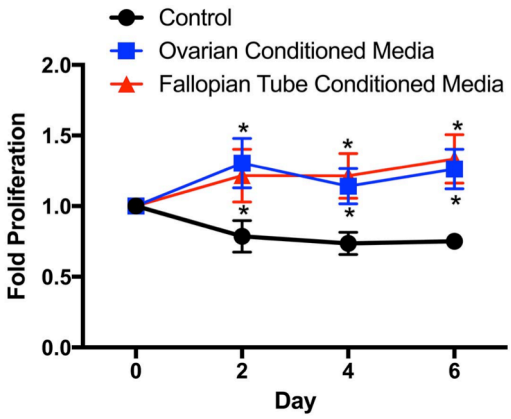


Figure 11. Proliferation of fallopian tube cells did not differ when stimulated with ovarian conditioned medium compared to oviductal conditioned medium.

KEY RESEARCH ACCOMPLISHMENTS:

- Cytokines were not pro-migratory, but a protein constituent was confirmed to be responsible and mass spectrometry based proteomics and size exclusion chromatography are being used to purify and identify the active protein(s).
- Fallopian tube cells can be grown on 3D collagen and have a similar downregulation of CTGF as high grade serous cancer lines.
- RNAseq was performed and indicated that the top KEGG pathways modified by adhesion, cytoskeleton, and MAPK signaling. The most highly up and down regulated genes will be functionally tested for their role.
- Fallopian tube cells were not transformed by H2O2 and were equally stimulated by both ovarian and oviductal medium.

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

- **Abstracts and presentations**
- Characterization of the Ovarian Secretome Identifies Proteins that Stimulate Migration of the Fallopian Tube Epithelium. (2015) Center for Biomolecular Science, University of Illinois at Chicago. Chicago, IL.

- Dean M, David D, and Burdette JE. (2016) Secreted Proteins from the Ovary Stimulate Migration of the Fallopian Tube Epithelium: A Mechanism for Initial Metastasis of High Grade Serous Cancer. College of Pharmacy Research Day. University of Illinois at Chicago. Chicago, IL.

- Dean M, Burdette JE. (2015) The role of the ovary in high grade serous cancer originating in the Fallopian tube epithelium. 7th Annual Illinois Symposium on Reproductive Sciences. University of Illinois Urbana-Champaign. Champaign, IL.

- Shaikh A, Dean M, Burdette JE. (2015) The role of cytokines in mediating migration of oviductal cells. University of Illinois Undergraduate Research Day: Posters Under the Dome. University of Illinois at Chicago. Chicago, IL.

- **degrees obtained that are supported by this award**
- **development of cell lines, tissue or serum repositories**
- **funding applied for based on work supported by this award**

CONCLUSION: Our results have identified that proteins that are not cytokines are the primary factor driving fallopian tube migration towards the ovary. We have also demonstrated that mutation of p53 blocks this signal, while PTEN loss enhances the migration towards the protein. RNAseq has revealed several new targets that are altered in response to adhesion to 3D collagen, which is a model of wounded ovarian surfaces that help to connect ovulation with the metastasis of fallopian tubes to the ovary. Lastly, while we hypothesized that the ovarian microenvironment would increase proliferation of fallopian tube cells, the secreted factors did not increase proliferation more than oviductal conditioned medium. This suggests that either additional mutations are required for fallopian tube cells to benefit from the ovary by proliferating or that the ovary may change as it ages to facilitate this biology.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

1 Yang-Hartwich, Y., Gurrea-Soteras, M., Sumi, N., Joo, W. D., Holmberg, J. C., Craveiro, V., Alvero, A. B. & Mor, G. Ovulation and extra-ovarian origin of ovarian cancer. *Scientific reports* **4**, 6116, doi:10.1038/srep06116 (2014).

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APPENDICES: N/A