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**TITLE: Immune Checkpoint Regulator in Ovarian Cancer Progression**

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> We hypothesize that CAF-derived MFAP5 can generate an immuno-suppressive microenvironment that suppresses CD8+ CTL activation by up-regulating CD47 expression in ovarian tumor cells and CD8+ CTLs and that inhibits CD8+ CTL trafficking through the extracellular matrix in the ovarian tumor microenvironment. A majority of experiments proposed under Major Goal 1 and a subset of experiments proposed under Major Goal 3 has been accomplished. Our results demonstrated that a marked inverse correlation between stromal MFAP5 expression and intraepithelial CD8+ T-cell density in high-grade serous ovarian tumor tissue samples. In addition, tumors developed in mice treated MFAP5-specific siRNAs or an anti-MFAP5 antibody had significantly lower CD47 expression levels than in those treated with the control siRNA or the control IgG antibody, respectively. Preliminary studies also demonstrated that markedly lower intratumoral CD8+ T cell densities in mice treated with MFAP5-specific siRNAs than the control siRNA. Taken together, MFAP5 silencing or blockade in ovarian tumor bearing mice activate tumor infiltrating CD8+ T cells and down-regulate CD47 expression in tumor tissue.					
<b>15. SUBJECT TERMS</b> Ovarian cancer, MFAP5, CD47, tumor microenvironment, CAF					
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## 1. INTRODUCTION

Although a subset of patients with advanced high-grade serous ovarian cancer (HGSC) survive more than 5 years, the vast majority have their cancers recur within 12-24 months after diagnosis and die of recurrent metastatic disease. The identification of predictive or prognostic markers for ovarian cancer is crucial for developing novel therapeutic targets and prolonging patient survival. Recent studies show that biomarkers expressed by specific stromal cell types in the tumor microenvironment may have prognostic value. The immune system is an important determinant of the tumor microenvironment; various immunologic gene products during ongoing inflammation create a favorable microenvironment for tumor growth and progression. Recent studies demonstrated that CD8<sup>+</sup> tumor-infiltrating lymphocytes are associated with improved overall survival and have been described in several solid tumors, including ovarian cancer. Nevertheless, the molecular mechanisms underlying the promotion or inhibition of CD8<sup>+</sup> lymphocyte infiltration in ovarian cancer are not fully understood. By analyzing the transcriptome profile of microdissected cancer-associated fibroblasts (CAFs) adjacent to tumor cells in ovarian tumor tissue from patients with HGSOCS, we identified a CAF gene signature associated with decreased intratumoral CD8<sup>+</sup> cytotoxic T cell (CTL) density. Among the genes in this signature, we found that high expression levels of CAF-derived microfibrillar-associated protein 5 (MFAP5), a 25-kD extracellular matrix glycoprotein with an RGD domain, has been shown to enhance the invasive potential of ovarian cancer cells through the  $\alpha_v\beta_3$ -dependent FAK/ERK/TNNC1 signaling pathway, were associated with decreased CD8<sup>+</sup> CTL density in the epithelial compartment of HGSOCS and with poor patient survival. Silencing MFAP5 in an ovarian cancer-bearing immuno-competent C57BL/6 mouse model significantly increased intraepithelial CD8<sup>+</sup> T cell density. Further functional studies showed that recombinant MFAP5 increased apoptosis in cultured CD8<sup>+</sup> T cells. Transcriptome profiling analysis showed a marked increase in the expression of CD47, a known immune checkpoint mediator that inhibits macrophage phagocytosis of tumor cells and deactivates CD8<sup>+</sup> T cells, in MFAP5-treated ovarian cancer cells and CD8<sup>+</sup> T cells. Correlative studies demonstrated significant correlations between higher CD47 expression in ovarian cancer cells, higher MFAP5 expression in CAFs, worse patient survival rates, and lower intraepithelial CD8<sup>+</sup> CTL density. We therefore hypothesize that CAF-derived MFAP5 can generate an immuno-suppressive microenvironment that suppresses CD8<sup>+</sup> CTL activation by up-regulating CD47 expression in ovarian tumor cells and CD8<sup>+</sup> CTLs and that inhibits CD8<sup>+</sup> CTL trafficking through the extracellular matrix in the ovarian tumor microenvironment. These effects, we hypothesize, lead to decreased intraepithelial CD8<sup>+</sup> CTL density and poor survival in patients with HGSOCS. MFAP5 blockade could therefore enhance CD8<sup>+</sup> CTL-mediated immune response and improve patient survival rates.

## 2. KEYWORDS

Ovarian cancer, MFAP5, CD47, tumor microenvironment, CAF

## 3. ACCOMPLISHMENTS

### a. What were the major goals of the project?

Specific Aim 1, Major Task 1: Evaluate the effect of MFAP5 blockade on intraepithelial CD8<sup>+</sup> CTL density (Months 1-12).

Specific Aim 1, Major Task 2: Evaluate and compare the effect of MFAP5 blockade on ovarian tumor metastasis and survival and T cell deprived mice (Months 10-24).

Specific Aim 2, Major Task 1: Evaluate the effect of MFAP5 on CD47 expression and intraepithelial CD8<sup>+</sup> CTL density (Months 12-16).

Specific Aim 2, Major Task 2: Evaluate whether CD47 mediates the effect of MFAP5 in preventing macrophage phagocytosis of ovarian cancer cells and on CD8<sup>+</sup> CTL activation (Months 1-30).

Specific Aim 2, Major Task 3: Evaluate whether CD47 mediates the effect of MFAP5 in inducing apoptosis in CD8<sup>+</sup> CTLs (Months 20-30).

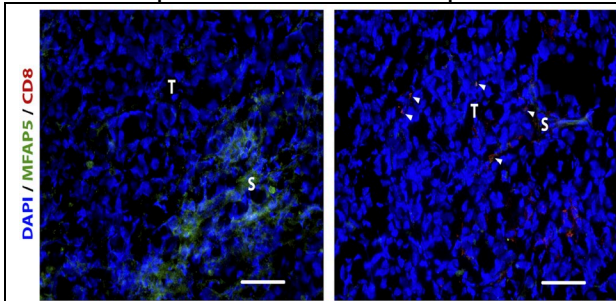
Specific Aim 2, Major Task 4: Evaluate the role of MFAP5 in inhibiting effector T cell trafficking through interstitial tissue space (Months 25-30).

Specific Aim 3, Major Task 1: Evaluate signaling pathways that mediate the effect of MFAP5 on CD47 expression in ovarian cancer cells and CD8<sup>+</sup> T cells (Months 24-30).

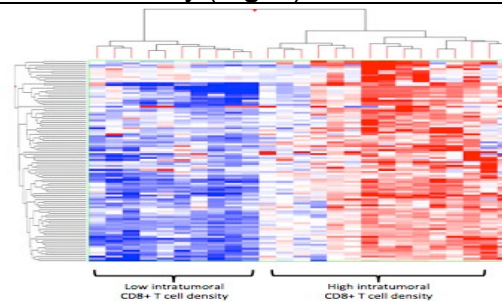
Specific Aim 3, Major Task 2: Evaluate the role of ovarian cancer cell-derived exosomes in up-regulating CD47 protein in CD8<sup>+</sup> T cells (Months 30-36).

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

**Specific Aim 1, Major Task 1** has been completed. We demonstrated that mice treated with MFAP5 specific siRNAs had markedly increased in number of CD8<sup>+</sup> T cells in the tumor tissues compared with those treated with the control siRNA (**Fig. 1**). In addition, transcriptome analysis on microdissected CAFs from human HGSC tissue with high (>median) and low (< median) CD8<sup>+</sup> T cell density showed higher stromal MFAP5 expression in tissue samples with low CD8<sup>+</sup> T cell density (**Fig. 2**).

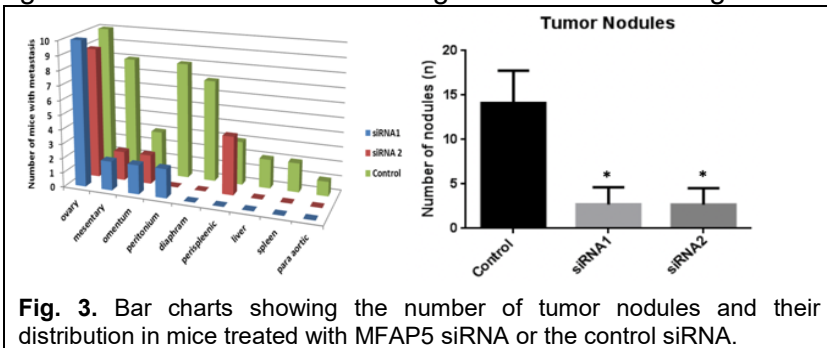


**Fig. 1.** Micrographs showing increased in CD8<sup>+</sup> T cells (arrowheads) in mice treated with MFAP5 specific siRNAs than the control siRNA.



**Fig. 2.** Heat-map showing differentially expressed genes in microdissected stromal tissue with high (>median) and low (< median) CD8<sup>+</sup> T cell density.

**For Specific Aim 1, Major Task 2**, we demonstrated that MFAP5 blockade suppressed ovarian tumor growth and metastasis. Silencing MFAP5 *in vivo* using siRNAs delivered by chitosan nanoparticles significantly



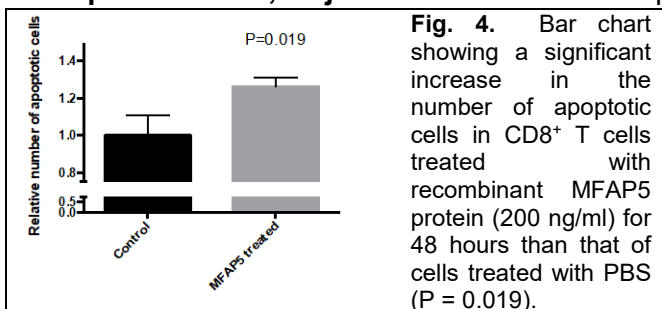
**Fig. 3.** Bar charts showing the number of tumor nodules and their distribution in mice treated with MFAP5 siRNA or the control siRNA.

lower the number of tumor nodules and the spread of the tumors to different internal organs of mice (**Fig. 3**).

In addition to using siRNA to inhibit MFAP5, OVCA432 ovarian cancer cell-bearing nude mice were injected with a new anti-MFAP5 blocking antibody that we have recently developed (Yeung TL et al., Clin Cancer Res, 2019, see section d) or the control IgG. The results showed that tumors developed in mice injected with the anti-

MFAP5 blocking antibody showed a significant decrease in CD47 staining intensity compared with those in mice treated with control IgG. These data suggest that CAF-derived MFAP5 may be the key mediator that up-regulates CD47 in ovarian cancer cells.

**Specific Aim 1, Major Task 3** has been completed and the results are summarized as follows:



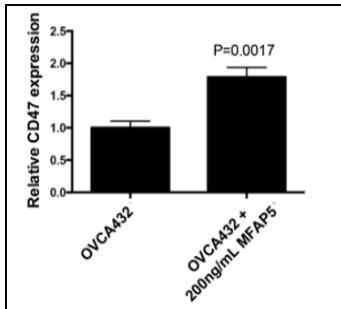
**Fig. 4.** Bar chart showing a significant increase in the number of apoptotic cells in CD8<sup>+</sup> T cells treated with recombinant MFAP5 protein (200 ng/ml) for 48 hours than that of cells treated with PBS (P = 0.019).

**MFAP5 up-regulated CD47 in CD8<sup>+</sup> T cells *in vitro*:**

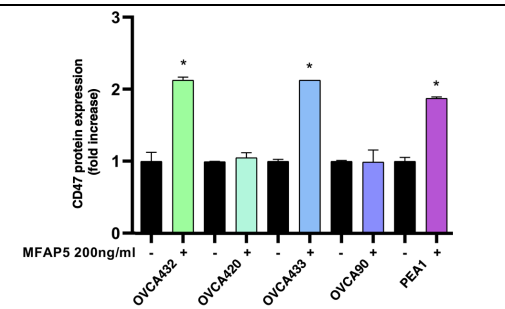
Our data demonstrated that MFAP5 up-regulates CD47 expression in CD8<sup>+</sup> T cells *in vitro*. Primary CD8<sup>+</sup> T cells (STEMCELL Technologies) isolated from peripheral blood mononuclear cells by negative immunomagnetic separation were treated with physiologic levels of MFAP5 (200 ng/ml) or control PBS. qRT-PCR analysis of CD47 was then performed. The results showed a significant increase in CD47 mRNA levels in CD8<sup>+</sup> T cells treated with MFAP5 compared with those treated with PBS (**Fig. 4**),

suggesting that MFAP5 can up-regulate CD47 in CD8<sup>+</sup> CTLs. Taken together, our studies suggest that CAF-derived MFAP5 regulates CD47, which is the only key immune checkpoint mediator associated with intratumoral CD8<sup>+</sup> CTL density in HGSCs and with poor survival in patients with ovarian cancer.

## MFAP5 induced CD47 mRNA and protein expression in ovarian cancer cells: qRT-PCR analysis on



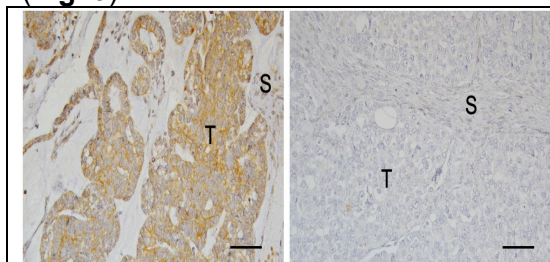
**Fig. 5.** Bar-charts showing higher levels of CD47 mRNA in MFAP5 treated cells than control cells.



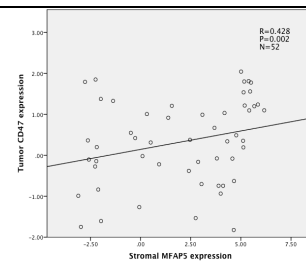
**Fig. 6.** Bar-charts showing higher levels of CD47 protein expression in MFAP5 treated cells than control cells.

ovarian cancer cell OVCA433 showed significantly higher levels of CD47 mRNA expression in cells treated with MFAP5 (200 ng/ml) than those treated with the control buffer PBS (**Fig. 5**). In addition, a majority of ovarian cells lines demonstrated significantly higher levels of CD47 protein expression when they were treated with MFAP5 than with PBS (**Fig. 6**). To further determine the role of CD47 in mediating the effect of MFAP5 in immune suppression correlation studies were performed. The results showed that CD47 expression levels in ovarian cancer cells

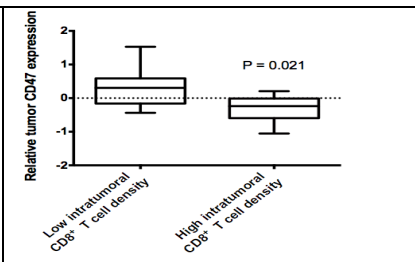
positively correlated with stromal MFAP5 expression levels (**Figs. 7 & 8**). In addition, significantly higher CD47 expression levels were also observed in ovarian tumor tissues with lower intratumoral CD8+ T cells densities (**Fig. 9**).



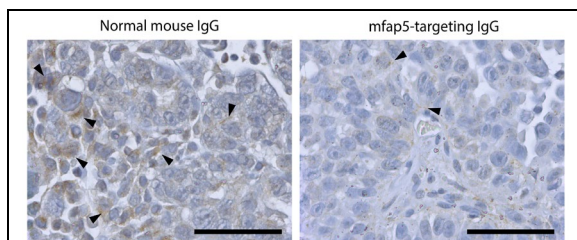
**Fig. 7.** Micrographs showing a HGSC tissue with high and low levels of CD47 expression. S, Stroma, T, tumor. Bar = 5 μm.



**Fig. 8.** Spearman analysis on stromal MFAP5 expression and CD47 expression in tumor cells.



**Fig. 9.** Box-plots showing significantly higher CD47 mRNA levels in tumors with low CD8+ T cell densities than those with high T cell densities.



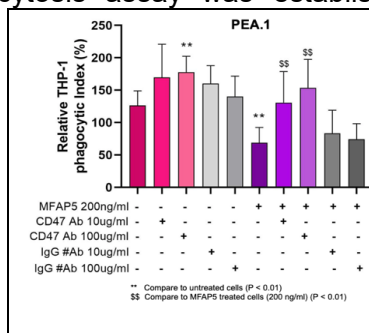
**Fig. 10.** Immunolocalization of CD47 showing lower CD47 protein expression in tumors (arrow heads) developed from OVCA432 bearing athymic mice treated with mfap5-targeting IgG compared with those injected with the control IgG. Bar = 50 μm.

## MFAP5 silencing down-regulated CD47 expression in ovarian cancer cell *in vivo*:

To determine whether MFAP5 overexpression is associated with increased CD47 in tumor tissue, OVCA432 ovarian cancer cell-bearing nude mice were injected with an anti-MFAP5 blocking antibody or the control IgG. The results showed that tumors developed in mice injected with the anti-MFAP5 blocking antibody showed a significant decrease in CD47 staining intensity compared with those in mice treated with control IgG (**Fig. 10**). These data indicate that CAF-derived MFAP5 may be the key mediator that up-regulates CD47 in ovarian cancer cells.

**Specific Aim 2, Major Task 2** has been completed. A

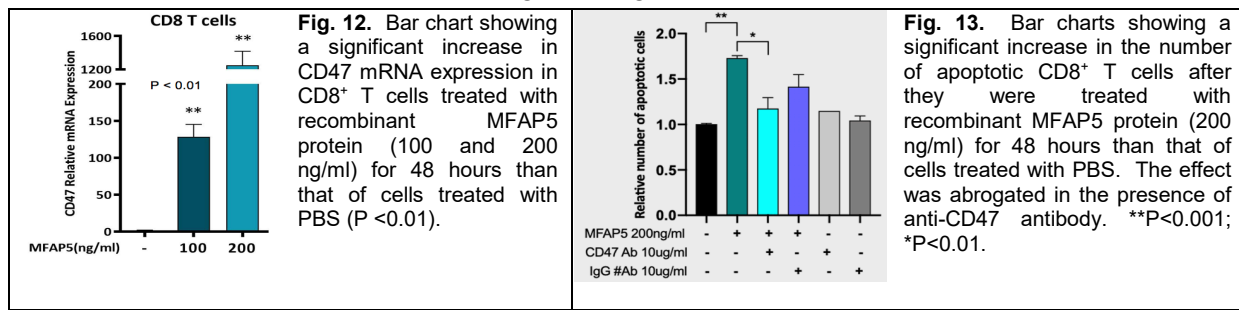
phagocytosis assay was established in which real-time imaging was used to quantify the number of



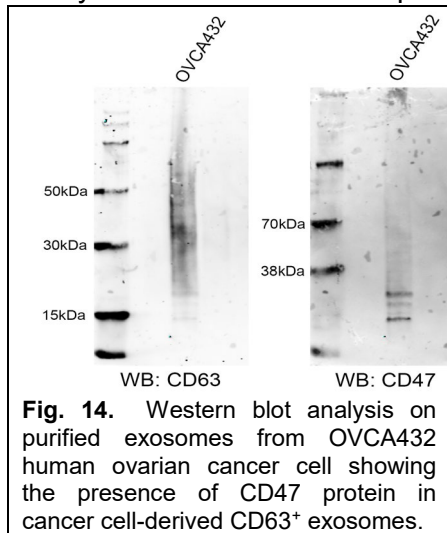
**Fig. 11.** Bar charts showing the promoting effect of MFAP5 on the phagocytotic potential of macrophages co-culturing with ovarian cancer cells PEA.1 in the presence or the absence of CD47 antibodies. Experiments were performed in triplicate.

of suggest that CD47 mediates the suppressor effect of MFAP5 on cancer engulfment by macrophages.

**Specific Aim 2, Major Task 3** has been completed. We demonstrated that MFAP5 induced apoptosis in CD8<sup>+</sup> T cells and the effect is mediated through up-regulation of CD47, a new immune checkpoint mediator.



Primary CD8<sup>+</sup> T cells isolated from peripheral blood mononuclear cells were treated with physiologic levels of MFAP5 (200 ng/ml) or control PBS. QRT-PCR analysis of CD47 was then performed. The results showed a significant increase in CD47 mRNA levels in



CD8<sup>+</sup> T cells treated with MFAP5 compared with those treated with PBS (**Fig. 12**), suggesting that MFAP5 can up-regulate CD47 in CD8<sup>+</sup> CTLs. Next, we demonstrated that MFAP5 treatment significantly increased the number of apoptotic CD8<sup>+</sup> T cells and the effect was abrogated in the presence of anti-CD47 antibody (**Fig. 13**). These findings indicate that CD47 mediates the pro-apoptotic effect of MFAP5 in CD8<sup>+</sup> CTLs.

**Specific Aim 3, Major Task 1**, computational analysis was performed on the promoter sequence of CD47 using *MatInspector*, TESS, and TFSEARCH software. We demonstrated that the CD47 promoter has a CREB and an NF-κB consensus binding sequence. Since promoter analyses of CD47 showed a potential CREB binding site and since our previous findings showing that binding of MFAP5 to α<sub>v</sub>β<sub>3</sub> integrin activates the calcium-dependent ERK/CREB signaling network in ovarian cancer cells<sup>1</sup>, we hypothesize that activation of the MFAP5/α<sub>v</sub>β<sub>3</sub> integrin/CREB signaling network also plays a role in up-regulation of CD47 in ovarian cancer cells and CD8<sup>+</sup> T cells.

**Major Task 2** is ongoing and will be completed in the no cost extension period. We demonstrated that CD47 protein cargo was detected in ovarian cancer cell-derived CD63<sup>+</sup> exosomes: To evaluate the regulation of CD47 protein expression in CD8<sup>+</sup> T cells by ovarian cancer cell-derived exosomes, exosome samples were isolated from conditioned media by ultracentrifugation at 4 °C, 24000rpm for 90 minutes. Purified exosome pellets were then resuspended in sterile PBS prior to Western blot analysis. The presence of CD47 protein was detected in purified exosomes from ovarian cancer cells (**Fig. 14**). These data suggested that in the tumor microenvironment, CD47 could be transferred from cancer cells to other cell types including CD8<sup>+</sup> T cells via secreted exosomes. Experiments are ongoing to test this hypothesis.

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

Nothing to report

**d. How were the results disseminated to communities of interest?**

A joint patent application #62/877,298 entitled “Microfibrillar-associated protein 5 (MFAP5)-specific antibody compositions and methods of use” has been filed together with Dr. Stephen Wong’s group at the Houston Methodist Hospital on 7/22/2019. Three manuscripts describing results related to the aims of the proposal have been published. They are listed as follows:

1. Leung CS, Yeung TL, Yip KP, Wong, KK, Ho SY, Mangala LS, Sood AK, Lopez-Berestein G, Sheng J, Wong ST, Birrer MJ, Mok SC. Cancer-associated fibroblasts regulate endothelial adhesion protein LPP to promote ovarian cancer chemoresistance. *J Clin Invest.* 2018;128(2):589-606. PubMed PMID: [29251630](https://pubmed.ncbi.nlm.nih.gov/29251630/); PubMed Central PMCID: [PMC5785271](https://pubmed.ncbi.nlm.nih.gov/PMC5785271/)
2. Yeung TL, Tsai CC, Leung CS, Au Yeung CL, Thompson MS, Lu KH, Freedman RS, Birrer MJ, Wong KK, Mok SC. ISG15 Promotes ERK1 ISGylation, CD8<sup>+</sup> T Cell Activation and Suppresses Ovarian Cancer Progression. *Cancers (Basel)* 10(12), 11/2018. e-Pub 11/2018. PMCID: PMC6316352.

3. Yeung TL, Leung CS, Yip KP, Sheng J, Vien L, Bover L, Birrer MJ, Wong STC, Mok SC. Anticancer immunotherapy by MFAP5 blockade inhibits fibrosis and enhances chemosensitivity in ovarian and pancreatic cancer. Clin Cancer Res. e-Pub 7/2019. PMID: 31332047.

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

A no cost extension has been approved due to the coronavirus outbreak. The project will end in 4/30/21. During the next reporting period (months 36-48), we will perform experiments according to those outlines in the proposal to further delineate the role of MFAP5 and CD47 in suppressing T cell activation and trafficking in ovarian tumor tissue. Specifically, experiments proposed in Specific Aim 3, Major task 2 will be completed as planned.

**4. IMPACT**

**a. What was the impact on the development of the principal disciplines of the project?**

Stromal genes serve as excellent therapeutic targets as they reside in a genomically stable (diploid) portion of the tumor. Our data suggest that CAF-derived MFAP5 may increase endothelial cell motility and permeability, and regulate immune checkpoint protein expression. If successful, our studies will demonstrate for the first time that a CAF derived protein up-regulated in ovarian cancer, MFAP5, plays a role in modulating angiogenesis and anti-cancer immunity, thus representing a stroma protein that can be targeted for ovarian cancer treatment selectively. Most importantly, this study will generate a novel monoclonal antibody for inducing anti-cancer immunity in specific subtypes of ovarian cancer, notably high grade serous ovarian cancer, providing a new treatment opportunity for those patients whose chemoresistant recurrent tumors do not respond to nivolumab or other immune checkpoint blockade agents. Further understanding of the molecular pathways associated with the biological effects induced by MFAP5 will uncover novel therapeutic targets for anti-angiogenic and immune therapies.

**b. What was the impact on other disciplines?**

Nothing to report

**c. What was the impact on technology transfer?**

A joint patent application #62/877,298 entitled "Microfibrillar-associated protein 5 (MFAP5)-specific antibody compositions and methods of use" has been filed together with Dr. Stephen Wong's group at the Houston Methodist Hospital (subcontract institution) on 7/22/2019. A humanized anti-MFAP5 antibody is under development and the feasibility of using the antibody as a therapeutic agent in ovarian cancer treatment will be evaluated.

**d. What was the impact on society beyond science and technology?**

Nothing to report

**5. CHANGES/PROBLEMS**

Nothing to report

**6. PRODUCTS**

A monoclonal anti-MFAP5 antibody which can recognize both human and mouse MFAP5 has been developed.

**7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

**a. What individuals have worked on the project?**

Nothing to report

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

No

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

**Organization Name:** N/A

**Location of Organization:** N/A

**Partner's contribution to the project:** N/A

**Financial support:** N/A

**In-kind support:** N/A

**Facilities:** N/A

**Collaboration:** N/A

**Personnel exchanges:** N/A

**Other:**

**8. SPECIAL REPORTING REQUIREMENTS**

Nothing to report

**9. APPENDICES**

Nothing to report