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Correlation of Clinical Data with Fallopian Tube Specimen Immune Cells and Tissue Culture Capacity

Satish Kumar Ramraj¹, Katie M. Smith², Naveena B. Janakiram³, Coralee Toal⁴, Ankita Raman⁵, and Doris Mangiaracina Benbrook^{1,2,*}

¹Gynecologic Oncology, Stephenson Cancer Center, University of Oklahoma Health Sciences Center, 975 NE 10th St, BRC1270, Oklahoma City, OK 73104

²Department of Obstetrics and Gynecology, University of Oklahoma Health Sciences Center, 800 Stanton L Young Blvd Suite 2400, Oklahoma City, OK 73104

³Department of Hematologic Oncology, 975 NE 10th St, BRC 1205, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

⁴University of Oklahoma Medical School, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

Abstract

Human fallopian tube fimbria secretory epithelial cells (hFTSECs) are considered an origin of ovarian cancer and methods for their culture from fallopian tube specimens have been reported. Our objective was to determine whether characteristics of the donors or surgeries were associated with the capacities of fimbria specimens to generate hFTSEC cultures or their immune profiles. There were no surgical complications attributable to fallopian tube removal. Attempts to establish primary hFTSEC cultures were successful in 37 of 55 specimens (67%). Success rates did not differ significantly between specimens grouped by patient or surgery characteristics. Established cultures could be revived after cryopreservation and none became contaminated with microorganisms. Two cultures evaluated for long term growth senesced between passages 10 and 15. M1 macrophages were the predominant cell type, while all other immune cells were present at much lower percentages. IL-10 and TGF- β exhibited opposing trends with M1 and M2 macrophages. Plasma IL-10 levels exhibited significant positive correlation with patient age. In conclusion, fallopian tube fimbria specimens exhibit a pro-inflammatory phenotype and can be

*Corresponding Author: 975 NE 10th St, Room BRC 1217A, Oklahoma City, OK 73104, Phone: (405) 271-5523, Doris-Benbrook@ouhsc.edu (Doris Mangiaracina Benbrook).

⁵Present address Department of OBGYN, University of Nevada, Las Vegas, NV-89102

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Authors Roles. SKR, KME, NBJ, CT and AR drafted various aspects of the manuscript and DMB collated their efforts, added her own efforts and wrote the final manuscript. SKR collected and processed the specimens and performed the culturing, Western blot and ELISA analysis and assisted in the data management and statistical analysis. KME, CT and DMB developed the plan for patient consent. CT and AR assisted in the specimen collection and transport. AR reviewed the patient charts for surgical data and evaluated the patient safety of the surgeries. NBJ directed the immune cell analysis. DMB designed the study, obtained the IRB approval and performed the statistical analysis.

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used to provide a source of hFTSECs that can be cultured for a limited time regardless of the donor patient age or race, or the type of surgery performed.

Graphical Abstract

Keywords

salpingectomy; human fallopian tube secretory epithelial cell cultures; fallopian tube fimbria; infiltrating immune cells; cytokines

1. Introduction

Epithelial ovarian cancer is often detected at late stage and is the most lethal of all gynecologic malignancies. Its etiology is poorly understood due to lack of early detection methods. The origin of the most common and lethal histology, high grade serous ovarian cancer, appears to include microscopic lesions that arise in the epithelial lining of the fimbria of the fallopian tube. This lining consists of proliferative secretory cells and non-proliferative ciliated cells.¹ Pre-neoplastic regions defined as dysplastic regions of continuous secretory cells with no intervening ciliated cells (SCOUTs)^{2,3} and serous tubal *in situ* carcinomas (STICs) have been found in BRCA mutant positive (BRCA+) women at high risk for ovarian cancer.^{4,5} Systematic analysis of fallopian tubes identified STICs in 38% of BRCA+ women.⁴ In a study of 55 consecutive cases of serous ovarian cancer not selected for BRCA status, 71% of the cancers involved the fallopian tube, and 48% of these cancer cases had a STIC which contained an identical p53 mutation to the cancer.⁵

Removal of fallopian tubes (bilateral salpingectomy) during surgery for benign gynecologic indications was shown to reduce the risk of ovarian cancer.⁶ A population-based retrospective cohort study reported that bilateral salpingectomy at the time of benign gynecologic surgery did not significantly increase the surgical time or risks for hospital readmission or blood transfusions.⁷ These surgeries provide a translational research opportunity to collect and study fallopian tube specimens for their role in ovarian carcinogenesis. Two reports have described growth of primary human fallopian tube secretory epithelial cell (hFTSEC) cultures from fallopian tube specimens, however the success rate of culture establishment attempts were not reported.^{8,9} Therefore, it is not known if characteristics of the patient, such as age, menopausal status or body mass index (BMI) or the type of surgical procedure employed to collect the specimen, such as laparoscopic versus abdominal or salpingectomy versus salpingo-oophorectomy, are associated with the success rate of culture establishment. In most studies, establishment of primary cultures from surgical specimens fall well below 100% with varying success rates

depending on the tissue of origin. The success rate of culture establishment is an important factor needed for appropriate study design.

Fallopian tube specimens could also be utilized to study how immune cell infiltrates contribute to tumorigenesis. Immune cells in fallopian tubes protect mucosal surfaces from infections, and also protect the sperm, embryo and fetus by controlling immune tolerance.^{10,11} Chronic inflammation caused by obesity however has been implicated in carcinogenesis.¹² Incremental increases in macrophages, but not T lymphocytes, were observed in STIC and high grade serous ovarian cancer lesions.¹³ A mixture of innate and adaptive immune cells were observed in fallopian tube tissue, with significantly higher T-helper and B cells and significantly lower macrophages in specimens from post-menopausal compared to pre-menopausal women.¹⁴ The immunohistochemical methods used in these studies however were only semi-quantitative and did not evaluate the immune cells differentiation or activation states. Circulating cytokines involved in immunosuppression and tissue repair, such as interleukin-10 (IL-10) and transforming growth factor- β (TGF- β), are also increased with obesity and age, and regulate the differentiation state of macrophages in association with carcinogenesis.^{15,16}

The purpose of this study was to determine if patient and surgical parameters were associated with the success rate of hFTSEC establishment and immune cell infiltrates of fallopian tube fimbria specimens. No complications attributable to the removal of fallopian tubes during surgery for benign gynecologic conditions occurred. We had a 67% success rate in establishing primary hFTSEC cultures independent of the patient and surgery characteristics. The fimbria microenvironment was predominantly pro-inflammatory with 20% of the cell population consisting of macrophages exhibiting the M1 differentiation state. This is the first publication to document the presence of T regulatory cells (Tregs) in fallopian tube tissue, and opposing associations of IL-10 and TGF- β with M1 and M2 macrophages that are consistent with the roles of these cytokines in regulating the macrophage differentiation state.

2. Materials and Methods

2.1 Patient Recruitment and Consent

This study was conducted under a University of Oklahoma Health Sciences Center (OUHSC) Institutional Review Board (IRB)-approved protocol (IRB #5563), which was approved on June 3rd, 2015. From June of 2015 until December of 2016, 91 healthy subjects were recruited from the OUHSC Gynecology and Urogynecology Clinics. Eligibility included patients scheduled for gynecologic surgery. Patients with cancer or who were less than 21 years of age were ineligible. Patients with only one remaining fallopian tube or indication for only unilateral salpingectomy were eligible for participation. Physicians in both clinics were introduced to trial, the inclusion and exclusion criteria, as well as the evidence and experience of removal of fallopian tubes in healthy subjects for ovarian cancer prevention at grand rounds and faculty meetings. Flyers describing the study, patient eligibility criteria and research personnel contact information were placed in the physician's workspace. Individual physicians informed the patients about the risks and benefits of salpingectomy as a part of the planned surgical procedure. Willing patients were then

consented by research staff and asked to fill out a brief questionnaire regarding their menstrual history and use of hormone therapy. Research staff reviewed the operating room schedules weekly and reminded the surgeon and laboratory research personnel about the specimen collection. Pre-operative nursing staff were trained to review records for each patient and to draw a purple-top vial of whole blood at the time of the patient's pre-operative lab draw or IV placement and place it on ice.

2.2 Specimen Processing

When the fallopian tubes were successfully removed, the operating room staff contacted the laboratory researcher using a phone number posted in each operating room for the duration of the study. When notified, the researcher on call would arrive at the surgical suite within 15 min, dress in surgical attire and enter the operating room where the fallopian tubes had been moved to a non-sterile field. The surgeon labeled the pad under the fallopian tubes to indicate the right and left side of the patient. Using scissors, the researcher made a longitudinal incision along the middle of the tube at the fimbriated end. Once the incision reached past the fimbria and the distal portion of the tube, a horizontal cut was made to separate a tissue sample consisting of half of the fimbria. For surgeries involving patients who had two tubes, one specimen was immediately placed in labeled containers containing DMEM/F-12 medium (Sigma Roche, 60%) containing antibiotic/antimycotic (Thermo Fisher, 1%), fetal bovine serum (FBS, Serum Source International, 36%) and DMSO (VWR, 4%) on ice, and the other was placed in a cassette which was then filled with OCT cryopreservation medium (Thermo Fisher). The cassette and a paper label with the study subject de-identified code written in pencil was wrapped in aluminum foil and submerged in liquid nitrogen. The remaining fallopian tube specimen was sent to pathology. All specimens were immediately transported to the laboratory. The blood was processed into multiple plasma aliquots, which were labeled and stored at -80°C .

2.3 Culturing of hFTSECs

Fresh fimbria specimens were processed for hFTSEC culturing in a biosafety cabinet under biosafety level 2 conditions using a published method⁸ with the following modifications. The specimens were minced with a sterile scalpel blade to achieve pieces of 1 mm, or small enough to pass through 5 ml serological pipette for efficient isolation. The DMEM-F12 medium was supplemented with fetal bovine serum instead of the more expensive Ultrosar G. A 0.2% of trypsin instead of regular 0.05% trypsin was used to release cells from collagen-coated tissue culture plates, which reduced the time needed for trypsinization from 15 to 3–5 minutes while increasing the percentages of live cells recovered. Inoculation density after trypsinization was $0.5\text{--}0.7 \times 10^5$ cells/ml in 6-well plate, at confluency cells density was $5\text{--}7 \times 10^5$ cells/ml.

The plate of cells was incubated at 37°C with 5% CO_2 and inspected the next day with a microscope for attached cell colonies or any signs of contamination, and replenished with fresh medium to remove cell debris. The culture plate was microscopically examined every two days and replenished with fresh medium. Those cultures which did not grow to confluency by day 6 were sterilized and discarded. Cultures that achieved confluency after about 6 days of incubation were categorized as successful and trypsinized. A portion of the

cells was placed onto a fresh culture plate, and the remaining portion was cryopreserved in DMEM/F-12 medium described above. After 5–6 passage, the hFTSECs could be cultured in a dish without the collagen coat and trypsinize using 1X trypsin.

2.4 β -galactosidase assay for cell senescence

Cellular senescence was evaluated by using the Senescence β -Galactosidase Staining Kit (Cell Signaling Technology) according to the manufacturer's instructions. Briefly, FTSECs were grown at different passages in 6 well plates. Once cells reached confluency, growth media was removed and the cells were rinsed with 1X Phosphate Buffered Saline (PBS). One ml of fixative solution was added to each well and incubated for 10–15 min. Plate was rinsed two times with 1X PBS. Further, 1 ml of the β -galactosidase staining solution was added and incubated at 37 °C overnight in an incubator that contained no water or CO₂ gas. The cells were observed under the microscope for the development of blue color and photographed.

2.5 Western blot

Total protein extracts were prepared from FTSECs or MCF-7 breast cancer cells (ATCC) using M-PER Mammalian protein extraction reagent (ThermoScientific) according to manufacturer's instruction. Western blots were performed according to a standard protocol¹⁷ and probed with a mouse monoclonal PAX8 antibody (Cell signaling) and a horse-radish peroxidase conjugated anti-rabbit (Cell signaling) secondary antibody and developed with Clarity Western ECL substrate (BioRad). The blot was also probed with an antibody to β -actin (Santa Cruz biotechnology) as loading control. ImageLab Software (Bio-Rad) was used to quantify the band intensities on digitized images of the blots.

2.6 Profiling Immune Cells

Fallopian tube specimens were dissociated for isolation of immune cells using a MACS Dissociator and a MACSmix™ Tube Rotator according to manufacturer's instructions (Miltenyi Biotech, USA). The resulting single cell suspensions were labeled with fluorescent tag-conjugated human antibodies (all were purchased from BioLegend) for Tregs (anti-CD4-PE, CD25-PerCP, Foxp3- Alexafluor 488), macrophages (anti- CD68-PE, IRF5-PE-Cy7, Stabilin-FITC), CD8a+ cytotoxic cells (anti- CD8-PE, CD25-pacific blue, perforin-APC) and Natural Killer cells NKs (anti-CD56-Alexafluor 488, CD16-PE, granzyme B-pacific blue, perforin-APC) and analyzed with a FACSCaliber and FlowJo software. As a negative control, cells were incubated with an isotype control antibody for the respective fluorescent tagged antibodies. Leukocytes were gated based on light scatter characteristics, followed by gating based on fluorescence minus controls. For Tregs, the double positive cells gated on CD4+-PE cells for PerCP and Alex flour 488 were used to calculate the percentage of total cells that were positive for all three markers (CD4+, CD25 and Foxp3). For NKs, the double positive cells gated on CD56-Alexafluor 488 cells for PE and pacific blue or perforin gave the percentage of total cells that are positive for three markers (CD56, CD16 and Granzyme B or CD56, CD16 and perforin). For CD8a+ cytotoxic cells, the double positive cells gated on CD8a+-PE for pacific blue and perforin gave the percentage of total cells that are positive for three markers (CD8a+, CD25 and perforin). The CD68-PE antibody was used to determine the gate on CD68 cells. The FITC positive cells gated on

CD68 gave the percentage of total cells that are positive for two markers (CD68 and Stabilin). The PE-Cy7 positive cells were gated on CD68 gave the percentage of total cells that are positive for two markers (CD68 and IRF-5). Ten thousand cells were evaluated per analysis and all immune cells were quantified as percentage of total cells in the dissociated specimen.

2.7 ELISA Analysis

Plasma specimens were rapidly thawed at room temperature and then immediately placed on ice. Levels of IL-10 or TGF- β were analyzed using commercial kits (R&D Systems, USA) and a SYNERGY H1 Multi Mode Reader (BioTech Instruments, USA). All specimens were evaluated in duplicate and concentrations were derived from a standard curve evaluated on the same plate in duplicate.

2.8 Statistical Analysis

Clinical data were entered into an Excel Spreadsheet. Weight gain per year since High School (approximately age 18) was derived by subtracting the patient weight at the time of surgery from her weight at the time of High School and then dividing by the patient age at the time of surgery. For continuous data, unpaired two-tailed T-tests comparing clinical data between the groups of patients whose cultures were successfully established versus not established were performed using Excel. GraphPad Prism version 9 was used to plot data and to perform Chi-square analysis and Fisher's exact test to evaluate the contingencies of ordinal clinical data with the success rates of culture establishment, Pearson Correlation of cytokine, immune cell and clinical data associations, and Spearman Correlation analysis of data that did not exhibit Gaussian Distributions. The D'Agostino & Pearson omnibus normality test was used to determine whether data sets exhibited Gaussian Distributions. P values of <0.05 were considered statistically significant.

3. Results

3.1 Surgeries and Outcomes

Ninety-one women were consented and enrolled in the study. Of these, 83 underwent their planned surgical procedure. The other patients did not proceed with surgery, did not have fallopian tubes present at the time of their surgery or withdrew from the study. There were 18 patients who desired elective sterilization and underwent laparoscopic bilateral salpingectomy. No complications were documented during the surgery and all patients had less than 30cc of intraoperative blood loss. Of the remaining patients, 43 had surgery for pelvic organ prolapse or other urogynecologic indications, and 22 had other benign gynecologic indications for surgery. Twenty-one of the patients had a minimally invasive hysterectomy with removal of fallopian tubes and conservation of the ovaries. This included vaginal, robotic and laparoscopic surgeries. An additional 4 patients had abdominal hysterectomies with salpingectomy. The remaining patients had salpingo-oophorectomy as the planned and completed procedure in addition to hysterectomy or other procedures. There were no complications recorded that were attributable to salpingectomy. The average blood loss for all procedures excluding sterilization was 136cc with the blood loss higher than

500cc occurring in two of the abdominal hysterectomy patients and 3 minimally invasive hysterectomy patients.

3.2 Correlation of Primary hFTSEC Culture Establishment Success with Clinical Data

The 55 specimens processed for hFTSEC culturing were chosen randomly. Thirty seven of these were successfully established as defined by their ability to grow to confluency beyond the first passage. On the first day of culturing, cells attached to the collagen coated plate as individual isolated colonies (Fig. 1A). Through day 3, “islands” of cells started to form, and by day 6 successfully established cultures had cells spread throughout the plate in a confluent manner. Western blot analysis of protein extracts from the first passage of two hFTSEC cultures documented the expression of PAX8, a marker of fallopian tube epithelial cells that is not expressed on ciliated or ovarian surface epithelial cells (Fig. 1C)^{18,19}. Cultures that did not achieve confluency on the plate by day 6 were categorized as not successfully established. Two cultures were evaluated for long term growth, and both senesced within 10–15 passages. Senescence was evaluated by β galactosidase assay (Fig. 1B), the morphology of the cells (Fig 1A) and the ability of cells to grow continuously. All established primary cultures were cryopreserved in a DMSO-containing medium at passage 1 or 2. Each of 6 attempts to thaw and re-culture the cells were successful.

The weight gain per year after High School Graduation did not differ between the groups of patients whose specimens could, and could not, be established in primary culture (Table 1 and Fig. 2A; unpaired two-tailed t-test, $p = 0.987$). Evaluation of weight using patient BMI, weight at time of surgery or weight at High School Graduation also did not differ between the specimens that were, and were not, successfully established (Table 1; unpaired two-tailed t-tests, $p > 0.05$). The patient age at time of surgery did not differ between established and not established cultures (Table 1, Fig. 2B; unpaired two-tailed t-tests, $p > 0.130$). The three oldest women whose specimens were used for culture establishment were 74 years old and hFTSEC cultures were successfully established from 2 out of these 3 specimens.

Success rates of culture establishment did not differ between specimens from pre- and post-menopausal women (Table 1, Fig. 2C; Chi-square = 0.078, $p = 0.779$) or between specimens from women with irregular periods associated with heavy bleeding and irregular periods associated with no bleeding (Table 1, Fig. 2D; Chi-square = 0.020, $p = 0.888$). Of the 37 cultures that were successfully established, 27 were from Caucasian patients, 8 were from African-American patients, 1 was from an Asian patient and 1 was from unknown race patient (Table 1). Of the 18 cultures that did not establish, 15 were from Caucasian patients, 2 from African American patients and one was from a Hispanic patient (Table 1). The cultures attempted from African American specimens had a higher rate of establishment (0.80) compared to cultures attempted from Caucasian patients (0.64), however the difference was not statistically significant (Fig. 2E; Fisher’s exact test, $p = 0.467$).

The types of surgeries utilized on the patients whose specimens were used for culturing cells were organized into four categories (Fig. 2F: 1) Sterilization: laparoscopic bilateral salpingectomy for sterilization. Historically these patients would have had just clipping or fulguration of the tubes; 2) Minimally Invasive Surgery (MIS) Elective: bilateralsalpingectomy (BS) at the time of otherwise indicated MIS surgery for gynecologic

or urogynecologic problems. Historically these patients would have had their tubes left in place; 3) Abdominal: abdominal surgery with BS or Bilateral salpingo-oophorectomy (BSO). These patients often would have had tubes left *in situ*. and 4) MIS Integral: MIS surgery with a or bilateral salpingo-oophorectomy (BSO). Even in a non-research setting, these patients would have had tubes removed. The rates of successful culture establishment did not significantly differ across specimens obtained from the four categories (Table 2, Chi-square = 5.2, $p=0.161$), however the cultures established from Sterilization surgeries trended to have higher rates of establishment compared to the other types of surgeries (Table 2, Fisher's exact test $p = 0.077$).

3.3 Profiles of Fallopian Tube Immune Cells and Circulating Cytokines

Flow cytometric analysis of cells from dispersed fimbria specimens revealed that the predominant cell type was M1 macrophages (20% of total cells), followed by Tregs (7%), CD8 cytotoxic T cells (3%), M2 macrophages (1%), perforin-positive NK cells (0.52%) and granzyme B-positive NK cells (0.13%) (Table 3). No significant associations between the percent immune cell types with patient characteristics were observed. IL-10 or TGF- β levels were measured in plasma samples from a representative subset of 40 patients chosen in a manner that incorporated the full BMI scale of the population in this study. Pearson Correlation analysis demonstrated negative trends between plasma levels of IL-10 and TGF- β with percent total macrophages and percent macrophages having the M1 phenotype in the fallopian tube specimens, and a positive trend between these two cytokines and M2 macrophages (Figure 3). Plasma IL-10 levels exhibited a significant positive correlation with patient age at the time of surgery (Figure 4, Spearman Correlation, $r = 0.407$, $p = 0.009$). No other significant correlations were observed.

4 Discussion

Our findings indicate that age of the patient is not a major factor in the utility of their fallopian tube fimbria specimens for growing hFTSEC cultures, and that patients up to at least 74 years of age can be used. The significance of being able to recruit multiple patients and establish multiple cultures independent of patient characteristics is that studies of multiple cultures from individual patients represent a more relevant model of clinical trials in comparison to studies of multiple replicates of a limited number of cell lines.

The higher rate of establishment from specimens collected from sterilization surgeries compared to all other surgeries, although only a trend, may reflect the lower average blood loss associated with this type of surgery compared to other surgeries (15.5 ml compared to 988 ml, respectively). A shorter time between separation from a blood supply and processing in the elective sterilization surgeries may also contribute to the higher viability of the cells in these specimens. During hysterectomies involving BS or BSO, the surgeon is likely to wait until all of the organs planned for removal have been surgically excised before giving the specimens to the research team. Therefore, the hysterectomy surgical procedure results in longer times between separation of the fallopian tube from the blood supply and processing of the tissue for research, while elective sterilization, which involves removal of the fallopian tubes only, results in shorter times between specimen removal and processing

for research. Another factor affecting the amount of time that the specimens are separated from the blood supply is that surgeries involving BSO remove the infundibulopelvic (IP) ligament. Removal of the IP ligament cuts off the fallopian tube from its major blood supply prior to surgical excision, whereas BS involves direct removal of the fallopian tube without IP ligament removal.

Although the primary cultures have a limited life span, this study demonstrated a reasonable success rate in establishing multiple primary cultures that can be cryopreserved and studied. There are multiple additional technical factors that could affect the success rate of hFTSEC culture establishment. The most common factor in our experience was the quality of fimbriae obtained from fallopian tube specimens. If fimbriae were small and unhealthy, then they were not successfully established as primary cultures. We also observed that inadequate mincing of tissue (e.g. when clumps of >1 mm were present) led to poor culture establishment. Proper rocking (~8 rpm) and time (36 hours) of the processed tissue during the digestive enzyme incubation was also important. Both shorter and longer incubation times led to poor culture establishment. Cells plated at low density after tissue processing were less likely to establish successfully. Thus, we recommend using smaller plates when the numbers of cells are limiting. While these primary hFTSEC cultures represent a normal-to-benign state, they can be immortalized to represent pre-neoplastic lesions and further transformed to represent cancer. One report demonstrated that transfection of hFTSECs with human telomerase reverse transcriptase (hTERT) plus SV40 large T and small T antigens could cause their immortalization (prevention of senescence) and allowed their subsequently transformed with oncogenes.²⁰ Other studies have shown that hFTSECs can be used to generate 3D cultures to evaluate carcinogenesis in a tissue microenvironment.^{9,21}

Similar to studies using immunohistochemical methods to profile immune cells in normal fallopian tube specimens^{13,14}, our more-quantitative flow cytometry analysis observed that the most prevalent cell type in the fallopian tube fimbria is the macrophage at 20% of all cells. We went further to profile the differentiation state of the macrophages and found an average M1/M2 ratio of 38 indicating a more inflammatory state. It is possible that the pro-inflammatory state was caused by the surgery. This study is the first to report the presence of Tregs in fallopian tube fimbria. Their presence at the second highest level of all immune cells evaluated may reflect a compensation to an inflammatory state associated with the M1 macrophages and other cytotoxic cells within the fimbria tissue.

Circulating levels of cytokines can impact tissue immune cell profiles and carcinogenesis. This study observed opposite trends between IL-10 and TGF- β with the percent of total macrophages and M1 macrophages compared to M2 macrophages. The positive trends of IL-10 and TGF- β with M2 macrophages is consistent with other reports and provides validation for our methods.^{15,16} In mouse studies, aging was associated with increased M1 macrophages in adipose tissue and livers, and increased M2 macrophages in bone marrow, spleen, lymph nodes, lung and muscle.²² In humans, aging is associated with development of low grade chronic inflammation (inflammaging) characterized by elevated levels of inflammatory cytokines, as well the IL-10 and TGF- β anti-inflammatory cytokines.²² The positive correlation observed between IL-10 and age in this study is consistent with an anti-inflammatory response to inflammation and with the observation that individuals with

genetic predisposition to produce high levels of IL-10 have a higher probability to survive longer²³, although earlier studies reported no association between IL-10 levels and age.^{24,25} Lack of significant associations between immune and patient parameters may be due to the low numbers of specimens evaluated.

This study provides preliminary data regarding the non-cancerous immune microenvironment in fallopian tubes that is consistent with a pro-inflammatory state (predominance of M1 macrophages) with some compensation by Treg cells (the second most predominant immune cell phenotype). The chronic pro-inflammatory state is associated with ovarian carcinogenesis, while an immune-tolerant state is associated with tumor promotion^{26–28}. These data provide a basis for comparison of normal fallopian tube tissue with STIC lesions and high grade serous ovarian cancer to determine if the microenvironments become more immune-tolerant with STIC development and progression.

The findings of this study can be used to guide the development of much-needed research of the role of the fallopian tube in ovarian carcinogenesis and of other gynecologic and fertility health issues.

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Abbreviations

BS	Bilateralsalpingectomy
BSO	Bilateralsalpingo-oophorectomy
hFTSEC	human fallopian tube secretory epithelial cell
hTERT	human telomerase reverse transcriptase
IL-10	interleukin-10
IRB	Institutional Review Board
MIS	Minimally Invasive Surgery
NK	natural killer
OUHSC	University of Oklahoma Health Sciences Center

SCOUT	secretory outgrowth
STIC	serous tubal in situ carcinomas
TGFβ	transforming growth factor-β
Tregs	T regulatory cells

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Highlights

- A 67% success rate in establishing cultures from fimbria specimens was achieved.
- This success rate was independent of patient and surgical characteristics.
- The predominant cell type in the fimbria specimens was the M1 macrophage.
- Tregs were the second most predominant immune cell type in the fimbria.
- Plasma IL-10 positively correlated with patient age.

Fig. 1. Characteristics of hFTSEC cultures

(A) Micrographs of cultures at different passages, bar (—) represents 100 μm. (B) **β-galactosidase assay of FTSECs at different passages**, bar (—) represents 100 μm (C) Western blot of PAX8 expression in hFTSECs protein extracts, PAX8 band intensities were normalized with the β-actin loading control band intensities and plotted in the graph shown for each specimen and MCF-7 cell line negative control.

Fig. 2. Fallopian tube culture growth success with respect to patient characteristics
 Comparison of culture establishment success with weight gain per year after high school (HS) (A), patient age at the time of surgery (B), menopausal status (C), irregular menstrual periods, race (D), race (E), and type of surgery (F).

Fig. 3. Correlation analysis of macrophages with plasma IL-10 and TGF-β cytokines
Macrophage and cytokine distributions in this subset of specimens were normally distributed. Graphical presentations, Linear Regression Analysis to draw the lines, and Pearson Correlation Analysis were performed by GraphPad Prism Software.

Fig. 4. Positive correlation of plasma IL-10 with patient age at time of surgery
IL-10 distribution in this subset of specimens was not normally distributed. Graphical presentations and Spearman Correlation Analysis were performed by GraphPad Prism Software.

Table 1

Comparison of Patient Characteristics

Patient's Profiles	Culture Did Not Establish	Culture Established	Establishment rate (%)
Race			
African-American	2	8	80
Caucasian	15	27	64
Asian	0	1	100
Hispanics	1	0	0
Unknown	0	1	100
Age (years)	51.35 ± 4.01 (range: 30–73 yrs)	45.7 ± 2.41 (range: 30–74 yrs)	
Hormone Status			
Pre-menopausal	10	22	
Post-menopausal	7 (0 by surgery)	13 (3 by Surgery)	
Irregular Period-Heavy bleeding	6	11	
Irregular Period-No bleeding	12	24	
Hormone Replacement	1	4	
Body Weight (lbs)			
Weight Gain/Year After High School	2.2 ± 0.299	2.21 ± 0.601	
Body Mass Index (BMI)	29.99 ± 6.1	26.05 ± 6.39	

* There were no significant associations between the patient parameters and the success rates of establishment ($p > 0.05$). Fisher's Exact Test was used for surgical group comparisons with $n < 5$ in either culture category. Chi-square was used for all surgical group comparisons that had $n > 5$ in both culture categories.

Table 2

Comparison of Culture Success with Type of Surgery

	Sterilization	MIS Elective	Abdominal	MIS Integral	All Surgeries ^a
Ave. Blood Loss	15.5 ± 12.7	323.7 ± 444.9	525 ± 247.5	139.6 ± 283.5	185.18 ± 328.37 ^b
Success Rate	10/11 (91 %)	9/15 (60%)	2/2 (100%)	15/26 (59%)	37/55 (67%)
Contingency of Success Rates ^c	Fisher's exact test, p = 0.077	Chi-square 0.42, p = 0.519	Fisher's exact test, p = 0.547	Chi-square 1.8, p = 0.178	Chi-square 5.2, p = 0.161

MIS : Minimally Invasive Surgery, Ave: Average in mls, Success Rate = Number of established cultures/Number of cultures that did not establish.

^aOne patient did not fit into any of the four surgical categories in this table.

^bAve. blood loss for all patients in this study: 187.64 ± 330.89 (n=83)

^cFisher's Exact Test was used for surgical group comparisons with n<5 in either culture category and Chi-square was used for all surgical group comparisons that had n>5 in both culture categories.

Table 3

Percent Total Cells with various Immune Phenotypes and Activation Status in Fimbria Specimens

	FT16	FT17	FT18	FT19	FT23	FT24	FT25	FT34	FT35	FT37	Ave	SE
Leukocytes												
CD8+ cytotoxic	4.28	0.19	0.93	4.08	0.1	0.72	5.98	0.21	8.34	2.53	2.74	0.87
NK perforin+	ND	0.01	0.24	0.29	0.31	0.56	1.43	0.44	0.92	ND	0.52	0.19
NK granzyme +	ND	0.08	0.01	0.17	0.25	0	0.19	0.35	1.92	ND	0.37	0.13
Tregs	ND	0.03	0.4	4.5	0.7	0.3	1.06	14.73	39.87	3.74	7.26	2.42
Macrophages												
Total	ND	4.70	85.0	26.50	15.6	6.52	15.80	5.68	38.4	53.5	27.97	9.32
M1	ND	0.05	53.8	20.45	15.03	4.32	14.77	5.07	21.35	44.94	19.97	6.66
M2	ND	2.57	0.46	1.73	0.16	0.15	0.42	0.42	1.55	1.67	1.01	0.34
M1/M2 ratio	ND	0.02	117.2	11.82	93.94	28.8	35.16	12.18	13.77	26.91	37.76	12.59

Leukocytes were gated based on light scatter characteristics, followed by gating based on fluorescence of antibody conjugates minus controls. CD8+ cytotoxic cells were identified as leukocytes positively labeled with antibodies to CD8, CD25 and perforin; Natural Killer (NK) cells were identified as leukocytes positively labeled with antibodies to CD56, CD16 and perforin, or to CD56, CD16 and granzyme B; T regulatory cells (Tregs) were identified as leukocytes positive labeled with antibodies for CD4, CD25 and Foxp3. Total macrophages are presented as % of total cells in the tissue dispersion, while M1 (CD68 and IRF5 positive) and M2 (CD68 and stabilin positive) macrophages are presented as % of macrophages. FT= Fallopian tube, Ave= Average, ND = Not Determined, SE = Standard Error.