Loss of CD73 Shifts TGF-β1 from Tumor Suppressor to Tumor Promoter in Endometrial Cancer

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Abstract

In cancer, transforming growth factor β (TGFβ) paradoxically functions as both a tumor suppressor and tumor promoter. In early-stage disease, TGFβ suppresses tumor growth by inducing cell cycle arrest and apoptosis. In late-stage disease, TGFβ promotes tumor cell invasiveness and metastasis. Often, mutations or genomic alterations in the TGFβ pathway are responsible for shifting the balance of TGFβ from tumor suppressor to tumor promoter. In endometrial cancer (EC), TGFβ pathway mutations and genomic alterations are rare, which suggests that the loss or gain of downstream targets are involved in shifting the balance. Another molecule, CD73, has also been implicated in EC. CD73 is a cell surface 5'nucleotidase that, in normal endometrium, protects epithelial integrity by increasing membrane F-actin. However, the loss of CD73 has also been found to increase EC progression.[1]

Recent work has found that TGFβ increases CD73 expression in normal endometrium and in HEC-1-A cells, a model of early-stage EC. The purpose of this study was to determine if loss of CD73 in EC shifts the action of TGFβ from a tumor suppressor to a tumor promoter. F-actin immunofluorescence was used to assess epithelial differentiation in normal murine endometrium. F-actin expression was increased in both glandular and luminal epithelial cells of mice treated with TGFβ. Additionally, F-actin and CD73 mRNA expression were positively correlated. These data suggest that TGFβ is associated with increasing CD73 and differentiation in normal endometrial tissue, and thus provide further evidence that TGFβ acts as a tumor suppressor in normal endometrium.

HEC-1-A cells were used to determine if inhibiting CD73 would increase TGFβ-mediated tumor-promoting activities in early-stage EC. MTT colorimetric assays showed that inhibiting CD73 with α,β-Methylene-ADP **(**AoPCP) increased cell proliferation in TGFβ-treated

cells. Phosphorylated SMAD2/3 and CD73 protein expression increased with TGFβ, indicating TGFβ increases CD73 through its canonical signaling pathway. Inhibiting CD73 in TGFβ-treated cells increased the protein expression of pro-metastatic transcription factor, Slug. Preliminary data also showed that inhibiting CD73 in TGFβ-treated cells decreased total F-actin. Taken together, these findings suggest that the loss of CD73 in EC shifts the action of TGFβ from tumor suppressor to tumor promoter. Future studies will focus on determining if the TGFβ-mediated increase of F-actin is via CD73. A better understanding of these and other molecular mediators of tumor progression can lead to the development of more targeted EC treatments.

Introduction and Background

Endometrial cancer (EC) is a malignancy arising in the epithelial lining of the uterus. It is the most common gynecological malignancy and fourth most common cancer diagnosis in women in North America. It is predicted that 63,230 new cases of EC will be diagnosed in 2018, with an expected 11,350 deaths.[2] Risk factors for EC include long-term exposure to high estrogen levels, obesity, increasing age, and a family history of EC or hereditary nonpolyposis colon cancer (HNPCC).[3] As with other cancers, EC can be treated with surgery, radiotherapy, and chemotherapy. However, unlike highly prevalent cancers such as breast cancer and lung cancer, there are currently no targeted therapies available for patients with EC. The lack of targeted therapies, coupled with an increase in incidence rate (due in large part to rising rates of obesity and an aging population), have added significantly to the clinical challenges of the disease. Although death rates for other cancers have decreased over recent years, death rates for EC have increased by 8% since 2008.[4] Accordingly, there is a great need for new treatment targets and diagnostic techniques for EC. Discovering genes and molecular pathways specific to endometrial cancer is imperative to finding more successful treatment strategies.[5]

Successful treatment is more likely if it occurs before EC has invaded the myometrium and metastasized to other parts of the body. After invading surrounding tissue, EC becomes more difficult to treat because EC cells tend to migrate through interconnected malignant glands.[1] Invasion of EC is measured with a progressive surgical staging system, where Stage I disease is limited to the endometrium and Stage IV disease involves disease metastasis to the lymph nodes and other tissues.[3] This staging system, and therefore the extent of invasion, is a good prognostic tool for five-year survival rates in EC patients. For Stage I disease, surgery is the most important and successful treatment option. The entire uterus can be removed by hysterectomy and, if

needed, the ovaries and other tissue in the abdominal cavity can also be removed.[3] The five-year survival rate for women diagnosed with Stage IA, and IB disease is 90% and 88% respectively.[6] Once EC has metastasized, however, there is no way to be sure that all the microscopic metastases have been removed. The five-year survival rates for women diagnosed with Stage IVA and Stage IVB EC are 17% and 15%, respectively.[6]

Postoperative radiotherapy can be used to treat any microscopic disease in the area surrounding the endometrium and in the lymph nodes. However, irradiation of other nearby organs, such as the bowel, bladder, and vagina, can cause serious long-term side effects in up to 10% of patients.[3] Chemotherapy and adjuvant hormone therapy can also be used to treat microscopic metastases, but there is currently no research that suggests these therapies improve the survival rate compared to surgery alone.[3] Chemotherapy and hormone therapy have also been known to cause severe side effects, as they are systemic treatments that can have detrimental effects on other parts of the body.[3]

Targeted therapies work by administering a drug that will interfere with a specific molecular pathway or protein that is unique to a particular type of cancer and even the individual patient's tumor. Because these treatments target only the tumor, they tend to be tolerated better than chemotherapy and radiotherapy.[7] There are currently no targeted therapy options for EC. Since surgery alone is not a definitive cure for advanced-stage EC, research is needed to develop targeted therapies to prevent the spread of microscopic metastases and recurrences of disease. Additional research is often necessary to discover mutations and proteins that can be the focus of a targeted drug, as the same tumor might express different mutations or different molecular targets from patient to patient. The work described in this thesis suggests that certain molecular players, such as CD73 and TGF-β1, may be potential candidates for targeted therapy in EC.

Transforming growth factor-β (TGFβ)

TGFβ has important functions in many cell signaling pathways involved in cell growth, tissue development, homeostasis, and immune system regulation.[8] TGF-β1 is a ligand for the TGFβ receptors. TGFβ is also indirectly involved in pathways that mediate apoptosis, epithelial to mesenchymal transition (EMT), cell migration, proliferation, differentiation and cell matrix formation, making it an important molecule in the control and prevention of tumor development.[8]

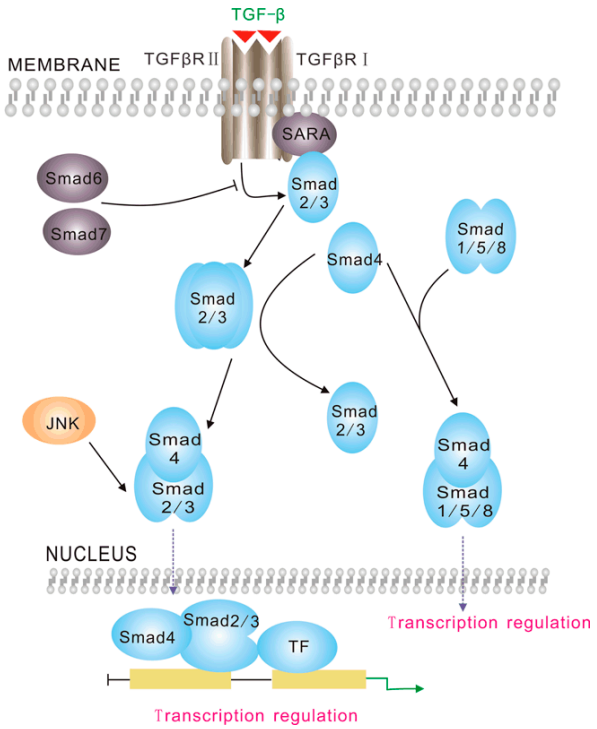

Figure 1: TGFβ pathway in normal tissue. A TGF-β ligand binds to a TGFβ receptor on the cell surface, which leads to the phosphorylation of Smad2/3, which then associates with SMAD4. This complex then translocates to the nucleus and interacts with various transcription factors of target genes. Figure was taken from https://www.sabbiotech.com/a-116-TGFb-smads-Pathway.html.

In cancer cells, TGF-β1 can act as both a tumor suppressor and promoter, depending on the type of cancer and stage of disease. This paradoxical behavior was first studied in murine skin cancer models. One study showed that TGF-β1 inhibited benign tumor growth in keratinocytes, but also promoted the progression of benign papilomas.[8] Cancer cells tend to lose

sensitivity to the effects of TGF-β1 as a proliferation suppressor through various mutations or alterations to downstream targets of the TGFβ pathway. Overexpression of TGF-β1 in many cancers is correlated with increased distant metastases and metastatic relapse, contributing to a poor overall disease prognosis.[8] One way TGF-β1 promotes tumor invasiveness and metastasis is through the facilitation of epithelial-to-mesenchymal transitions (EMT), which allows epithelial cells to lose cell-cell adhesions (essential to maintaining epithelial integrity and differentiation), and gain motility and invasive properties.[9] TGF-β1 promotes EMT through Smad-dependent transcriptional effects on tumor-promoting genes, such as *Snail*, *Slug*, and *Twist*.[9]

Alterations to the TGFβ pathway that cause an increase in tumor-promoting activity have been correlated to disease progression in many cancers.[8] As a result, the TGFβ pathway has become a popular therapeutic target for drug design. Inhibitors of the TGFβ pathway target the production of TGF-β1 and TGFβ receptors. Other TGFβ pathway therapies target the immunosuppressive effects in cancers such as glioma, melanoma, and renal cell carcinoma.[9] In EC, mutations to the TGFβ pathway are rare, which suggests downstream targets are likely involved in the shift of TGF-β1 from cancer suppressor to cancer.[8]

Ecto-5'nucleotidase (CD73)

CD73 is a cell surface 5'nucleotidase that generates adenosine by dephosphorylating adenosine monophosphate, a breakdown product of adenosine triphosphate (ATP). In tissue injury or disease, ATP is released to the cell surface. CD73, in combination with CD39, an upstream nucleotidase, generates adenosine to protect tissues. For example, in response to inflammation in the colon[10] and hypoxia in the endometrium[1], CD73 and extracellular adenosine are crucial for maintaining epithelial barrier function and epithelial integrity.

CD73-generated adenosine is a protective response that also promotes immunosuppression, angiogenesis, mucosal hydration, and ischemic preconditioning.[11] Notably, some of these responses (e.g. immunosuppression and angiogenesis) are advantageous to cancer cells. For example, breast tumors are highly enriched with stromal and fibrotic cells, which are the main contributors to CD73 expression, rather than the actual tumor cells.[1] Therefore, it is unlikely that CD73 maintains the epithelial barrier or epithelial integrity in normal breast epithelial tissue, because the epithelium is not the main contributor to CD73 expression. In contrast, endometrial tumors are less immunologic and are not comprised of large numbers of stromal and fibrotic cells. In late-stage, poorly differentiated EC, maintaining cell-to-cell adhesions is not advantageous to tumor progression and metastases. Given that CD73-generated adenosine is essential to maintaining barrier function/epithelial integrity, the loss of CD73 appears to be an important part of EC progression, as breaking down epithelial cell-cell adhesions is required for cell proliferation and metastases.[1]

Previous studies by Drs. Bowser, Broaddus and colleagues at the University of Texas MD Anderson Cancer Center reported that CD73 is significantly down-regulated in poorly-differentiated and advanced-stage EC.[1] Given that CD73 expression in Stage I EC is similar to normal endometrium, these findings suggest that the loss of CD73 promotes disease progression. The studies demonstrated that inhibiting CD73 in early-stage EC models increased EC cell migration and invasion.[11] They also demonstrated that CD73 prevented disease progression by its generation of extracellular adenosine to reform cell-to-cell adhesions, thereby regaining epithelial integrity and differentiation.[1]

Both TGF-β1 and CD73 have roles in the protective barrier function of epithelial cells. Previous research by Bowser et al. found that CD73 is involved in maintaining cell-to-cell

contact in response to stress through the regulation of F-actin polymerization via lengthening cell filopodia in HEC-1-A cells, a model of early-stage EC.[11] TGF-β1 has also been found to increase barrier function in epithelial cells of the colon via Smad signaling and upregulation of tight junctions.[12] Unpublished studies in the Broaddus laboratory also showed that CD73 expression in the normal endometrium and in early-stage models of EC, such as in HEC-1-A cells, is increased by TGF-β1. TGFβ acts as a tumor suppressor in early-stage disease by promoting epithelial differentiation and inhibiting cancer cell proliferation.[13] However, TGF-β1 has also been reported to be a tumor promoter in EC.[14] Given the correlation between levels of TGF-β1 and CD73, and the paradoxical roles of TGF-β1, I questioned whether the loss of CD73 in EC shifts the TGFβ pathway from acting as a tumor suppressor to promoting tumor progression. *I hypothesized that TGF-β1-mediated increase of CD73 in normal endometrium would be correlated with increased epithelial differentiation through increased F-actin expression. I also hypothesized that inhibiting CD73 in HEC-1-A cells would increase TGF-β1-mediated tumor-promoting activities, such as increased cell proliferation.*

Methods

Mice

Female C57BL/6 mice were purchased from the Experimental Radiation Oncology Animal Core at the University of Texas MD Anderson Cancer Center. Mice estrous cycles were synchronized, and the mice received either 10 μg/kg TGF-β1 (n=11) or BSA/HCl (n=11) by intraperitoneal injection when they were in the proestrus phase of their cycles, which was determined via vaginal smear. Twenty-four hours after injection, mice uteri were removed and stored at -80°C for RNA and histology studies. Vaginal smears were performed on mice prior to removing the uteri to confirm that the mice had entered the estrus phase 12-24 hours after pro-estrus. Pro-estrus/estrus mice were used as these phases allow the study of endometrial epithelial cell differentiation, which does not occur in metestrus and diestrus.[15]

HEC-1-A cell line

The HEC-1-A cell line is a model of early-stage endometrial cancer. This cell line was derived from a stage IA, noninvasive endometrial tumor, which was completely maintained to the endometrium.[16] HEC-1-A stock plates (100 mm) were maintained in McCoy's 5A medium, 10% fetal bovine serum, and 1x penicillin/streptomycin. Stock plates were incubated in a cell culture incubator maintained at 37°C and 21% O_2, 5% CO_2. Cells were allowed to grow to 90% confluence and removed from 100 mm plates for experiments using 0.05% trypsin. Cells were then plated into a 6-well plate at $5x10^5$ cells per well for Western blot and into 96-well plate at 8,000 cells per 100 μL for MTT assay.

Immunofluorescence

Mouse uteri for immunofluorescence studies were selected based on CD73 mRNA levels. Nine (n=9) mice with low CD73 mRNA expression and one internal control mouse with high

CD73 (#65) were chosen to represent the BSA/HCl group. Ten (n=10) mice with high CD73 mRNA expression and one internal control with low CD73 (#22) were chosen to represent the TGF-β1 group. Internal controls were used to validate the specificity of a possible correlated change in F-actin. Therefore, if CD73 mRNA correlates with F-actin, CD73-low-tissue would have low F-actin and CD73-high-tissue would be expected to have high F-actin regardless of treatment. In assessing F-actin intensity, I was blinded to the treatment groups/mice cryosections, and an epithelial marker, E-cadherin (double staining of cryosections; E-cadherin and F-actin) was used to assist in identifying epithelial cells from other cells, such as stromal cells.

Mice uteri were processed as cryosections and stored at -80˚C. For immunofluorescence experiments of F-actin, cryosections were warmed to room temperature, fixed with 4% paraformaldehyde for 20 minutes, and washed three times for five minutes in 1xPBS. The uterine tissues were permeabilized with 0.1% Triton X-100, washed three times for five minutes in 1xPBS, and non-specific staining blocked with background snipper for one hour. The nuclei were stained with DAPI, at a dilution of 2 µL DAPI per 1,000 µL of 1xPBS. F-actin was stained with Alexa-488 conjugated phalloidin (green fluorescence), at a dilution of 5 µL phalloidin per 200 µL of 1M PBS.

Immunofluorescence experiment slides were viewed with an Olympus X81 microscope at 20x and 40x magnifications. Exposure was kept constant for each magnification level at 32 ms for 20x magnification and 27 ms for 40x magnification. Images were taken of both glandular and luminal tissue. Relative F-actin expression of glandular and luminal epithelial cells was determined by measuring the intensity of the fluorescence using defined regions of interest

(ROIs) and CellSens dimension software. To control for differences in F-actin expression in glandular and luminal epithelial tissue, data were analyzed separately.

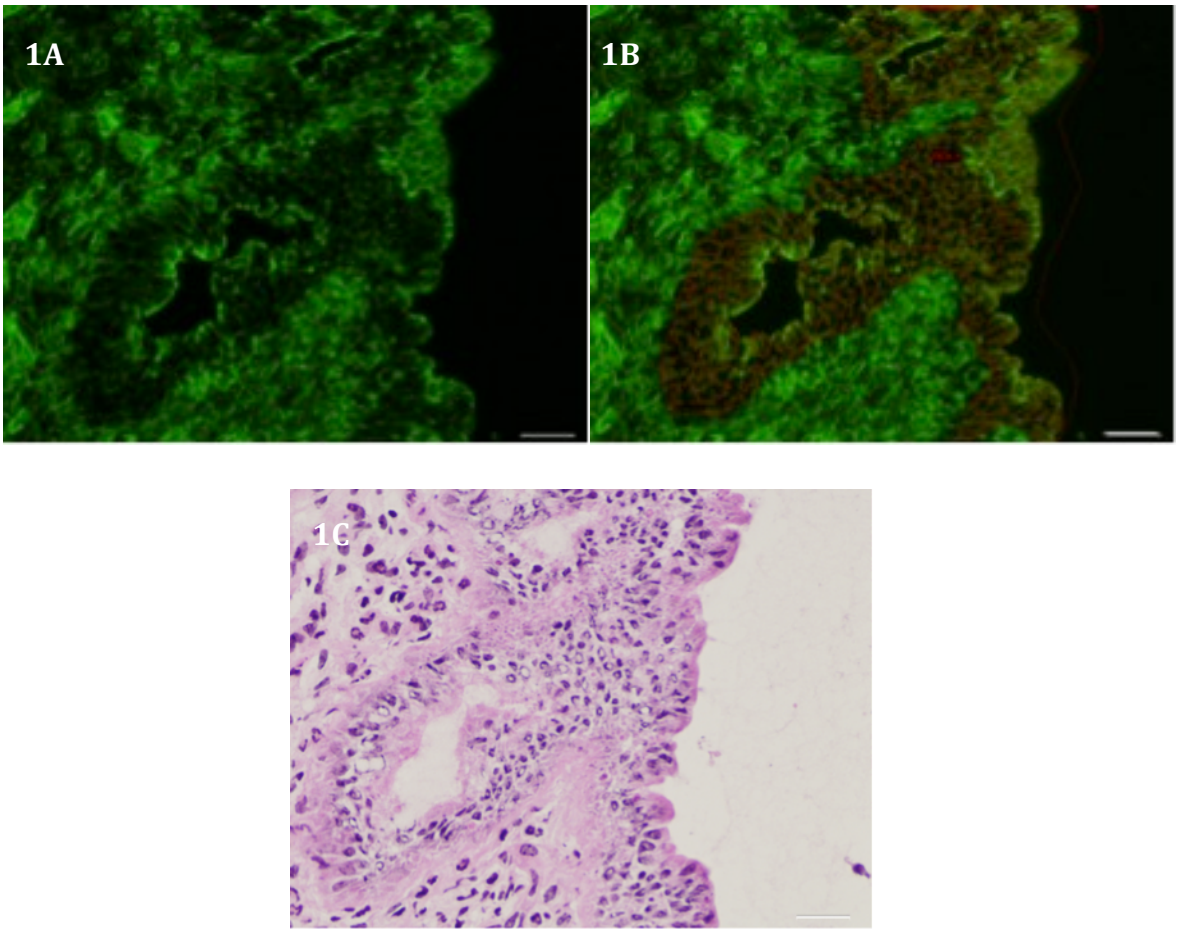

Figure 1. F-actin expression in normal murine endometrium treated with BSA/HCl at 40x magnification. Figure 1A demonstrates green F-actin immunofluorescence with arrows indicating luminal and glandular epithelial cells. Figure 1B demonstrates the red ROI selection used to measure immunofluorescence with CellSens software. Figure 1C shows the same slide stained with hematoxylin and eosin.

Western blot

HEC-1-A cells were maintained in McCoy's 5A medium (3ml per well) and incubated at 37˚C. Cells were serum-starved for 24 hours using Opti-MEM without fetal bovine serum (FBS). After 24 hours, the cells were pretreated for one hour with 100 µM AoPCP or Opti-MEM medium alone. Then, cells were treated with BSA/HCl (control), 2.5 ng/mL TGF-β1,

BSA/HCl+100 μM of AoPCP, or 2.5 ng/mL TGF-β1+100 μM AoPCP in Opti-MEM without FBS. AoPCP inhibits the enzyme activity of CD73. Cells were incubated at 37°C for 24 hours before protein isolation.

The cells were then washed two times with cold 1xPBS. Cell lysis buffer (1x) containing 1x protease and phosphatase inhibitors was added to the wells of the 6-well plates (200 μL per well). Cells were scraped off the plates, pipetted into microcentrifuge tubes, and centrifuged at 1,500 RPM for five minutes. The cell lysates were incubated on a shaker at 4˚C and then centrifuged at 12,000 RPM for ten minutes at 4˚C. The supernatant containing the proteins was transferred into new microcentrifuge tubes. The concentration of the proteins was measured using the Bradford assay and a spectrophotometer to ensure the same volume (20 μg) of protein was added to each well. Proteins were loaded at a total volume of 20 μL into the wells of 10% polyacrylamide gels. Gels were run at 70 V for 10 minutes and then at 100 V for 90 minutes. Proteins were transferred onto a PDVF membrane. Nonspecific antibody binding was blocked with 5% non-fat dry milk in PBST for one hour. Primary antibodies in 5% non-fat dry milk were added and the membrane and incubated overnight on a shaker. Afterwards, secondary antibodies in 5% non-fat dry milk were added and incubated for one hour. Enhanced chemiluminescence mix (ECL) was prepared and the membrane incubated for 1-2 minutes. A cassette and film were used to expose the protein on the membrane in a dark room.

MTT colorimetric assay

HEC-1-A cells were plated in 100 μL McCoy's 5A medium in 96-well plates. One plate was set up for each of six time points (0 hours, 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours). Color absorbance was measured at each of the time points to determine cell metabolic activity, and therefore, cell viability. At each of the time points, 20 μL of MTT solution were

added to each well. Cells were incubated for four hours at 37 ˚C in the dark. Then, 200 µL of acidic isopropanol were added to each well, and plates were placed on a slow-moving shaker and incubated at 37 ˚C in darkness for an additional hour to allow for the reduction and color change of the MTT solution. Absorbance was measured at 570 nm with a spectrophotometer plate reader.

Statistical Analysis

GraphPad software was used to analyze data. Because there was a difference in F-actin expression between luminal and glandular epithelium, data were analyzed separately for each cell type. Independent t-tests were run to analyze the difference in F-actin expression between tissues treated with TGF-β1 and BSA/HCl within luminal epithelium and glandular epithelium. A one-way ANOVA was run to determine the differences in protein expression among each treatment group and cell type. A linear regression was used to correlate CD73 mRNA expression with F-actin expression across BSA/HCl and TGF-β1 treatment groups.

Results

TGFβ-mediated increase of CD73 correlates with increased F-actin

Previous unpublished research done in the Broaddus laboratory found that TGFβ increases CD73 expression in early-stage EC models. Since CD73 promotes epithelial integrity in early-stage EC, it is possible that TGFβ's increase of CD73 in early-stage EC is what achieves epithelial differentiation.[1] The unpublished results showed that intraperitoneal injection of TGF-β1 in C57BL/6 mice significantly induced CD73 expression (data not shown). To assess for a positive correlation between CD73 and epithelial differentiation, F-actin expression was analyzed in endometrial epithelial cells of normal murine uterine cryosections. Uterine cryosections were chosen based on previously collected CD73 mRNA levels in individual mice.

F-actin intensity was assessed in epithelial cells of the uterine cryosections using ROI selections. Figure 3 shows the mean F-actin expression for mice in each treatment group. F-actin expression in epithelial cells of the endometrium was significantly higher ($p<0.001$) in TGF-β1 treated mice (mean=49.41) as compared to BSA/HCl treated mice (mean=39.84).

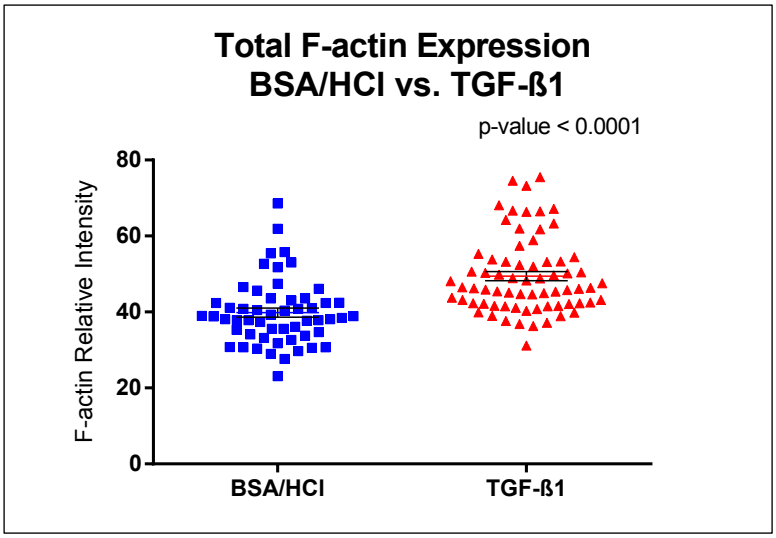

Figure 2: Total F-actin expression means for BSA/HCl (n=11) and TGF-β1 (n=11) treatment groups. Symbols indicate individual ROI selections from slides. F-actin expression is significantly higher in mouse uteri treated with TGF-β1 (BSA/HCl mean= 39.84, TGF-β1 mean=49.41, d.f.=121, $p<0.001$).

Since F-actin expression differed between glandular and luminal epithelial tissue in mouse uteri, F-actin intensity was analyzed separately to account for this difference. F-actin expression was significantly higher ($p<0.001$) in endometrial tissue treated with TGF-β1 in both luminal and glandular epithelium. A one-way ANOVA and Tukey's multiple comparison tests were run to determine differences between treatment groups. There was a significant difference in mean F-actin expression between all groups except luminal TGF-β1 and glandular BSA/HCl.

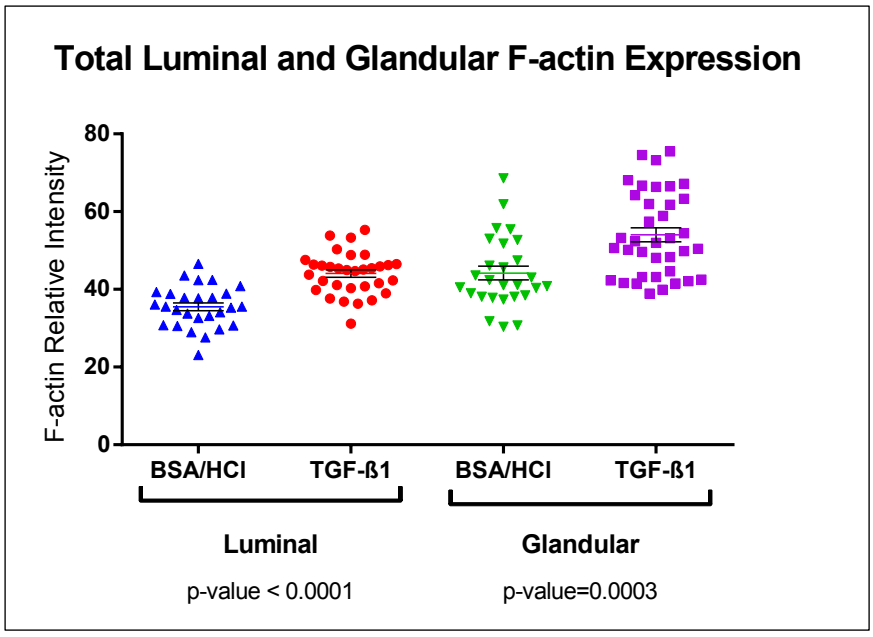

Figure 3: F-actin expression in mouse uteri. Symbols indicate individual ROI selections from slides. F-actin expression increased in both glandular and luminal epithelium in mice treated with TGF-β1 (Luminal BSA/HCl mean=35.50, luminal TGF-β1 mean=44.07, glandular BSA/HCl mean=44.18, glandular TGF-β1 mean=54.03, d.f.=119, $p<0.0001$)

F-actin expression was compared to CD73 mRNA levels collected from all mice. A linear regression was run to determine the relationship between CD73 mRNA levels and F-actin expression. The internal controls were removed from both the BSA/HCl and TGF-β1 treatment groups for this analysis. High F-actin expression was positively correlated with high CD73 expression, suggesting TGF-β1 is involved in increasing epithelial differentiation in normal

endometrium. Mice that were treated with TGF-β1 and had high levels of CD73 mRNA also had high F-actin expression. Mice that had lower CD73 mRNA had lower expression of F-actin.

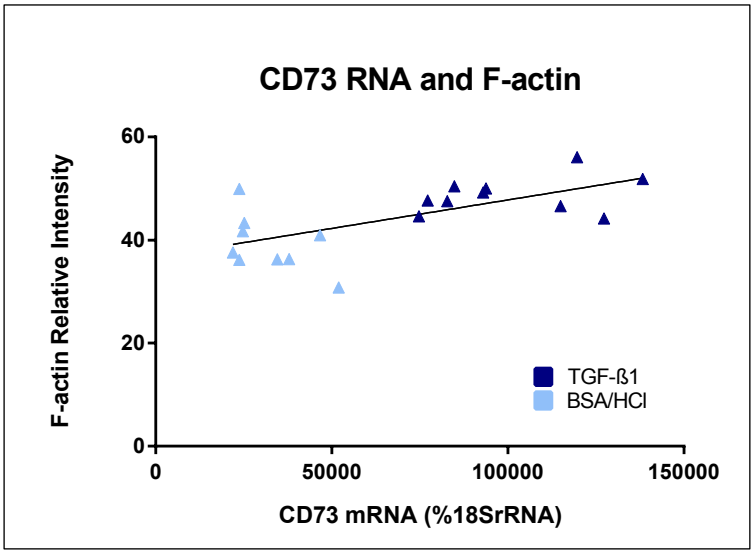

Figure 4. Correlation analysis of F-actin expression and CD73 mRNA expression of individual mice. Mice expressing high F-actin also expressed high levels of CD73 mRNA. Individual mice are represented by triangles. Color indicates treatment group. (Pearson's r=0.6543. R^2=0.4281. p=0.0024.)

Inhibiting CD73 in HEC-1-A cells increased TGFβ-mediated tumor promoting activities.

In order to assess the proposed tumor-promoting activities of TGF-β1 in HEC-1-A cells, western blots were performed to study protein expression. Cells were placed in one of four different treatment groups: (1) treatment with BSA/HCl, (2) treatment with TGF-β1, (3) treatment with BSA/HCl+AoPCP, and (4) treatment with TGF-β1+AoPCP. AoPCP was used as an enzyme inhibitor for CD73, and GAPDH was used as a control for equal loading of gel samples. Cells that were treated with TGF-β1 and TGF-β1+AoPCP showed an increase in CD73 protein expression, indicating that TGFβ's control of CD73 does not work in a positive feedback loop. Cells that were treated with TGF-β1 and TGF-β1+AoPCP showed an increase in pSmad 2/3 and a decrease in Smad 2/3. When CD73 was inhibited by AoPCP in cells treated with TGF-β1, there was an increase in expression of pro-metastatic transcription factor, Slug.

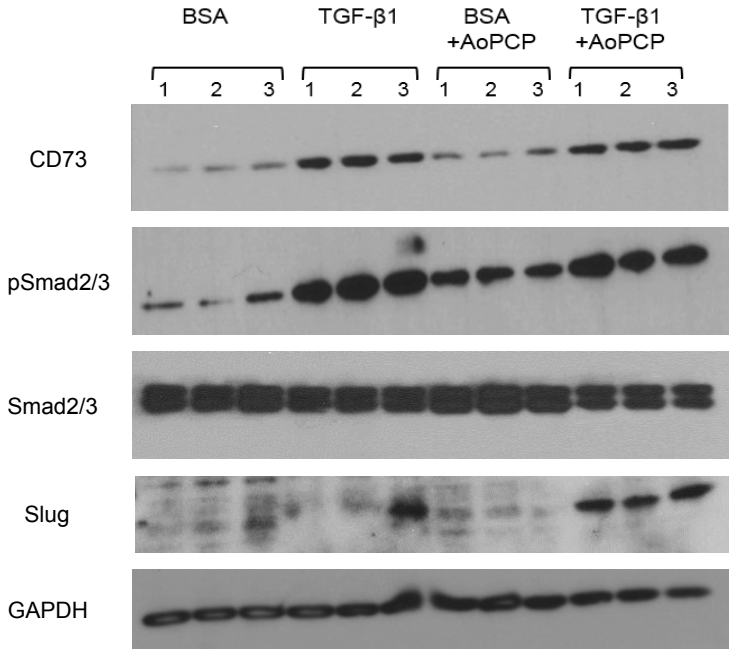

Figure 5: Western blots for HEC-1-A protein expression. Cells were treated with BSA, TGF-β1, BSA+AoPCP, and TGF-β1+AoPCP. CD73, pSmad2/3, and Slug proteins were analyzed, using GAPDH as a control.

Data from Western blots were analyzed using ImageJ densitometry software. Results for densitometry analysis are shown in Figure 6. There was no significant difference in CD73 protein expression for cells treated with TGF-β1 and cells treated with TGF-β1+AoPCP, indicating that there may not be a feedback loop present from CD73 to TGF-β1 in this pathway. There was a significant difference in Psmad2/3 protein expression between BSA and TGF-β1 treatment groups ($p=0.001$), and BSA and BSA+AoPCP treatment groups (0.01). However, there was no significant difference in expression between TGF-β1 and TGF-β1 +AoPCP treatment groups. There was a significant difference in Smad2/3 protein expression between BSA and TGF-β1 treatment groups ($p=0.01$) and between BSA+AoPCP and TGF-β1+AoPCP treatment groups ($p=0.001$). Slug protein expression was significantly higher ($p=0.003$) in cells treated with TGF-β1 and AoPCP compared to all other treatment groups.

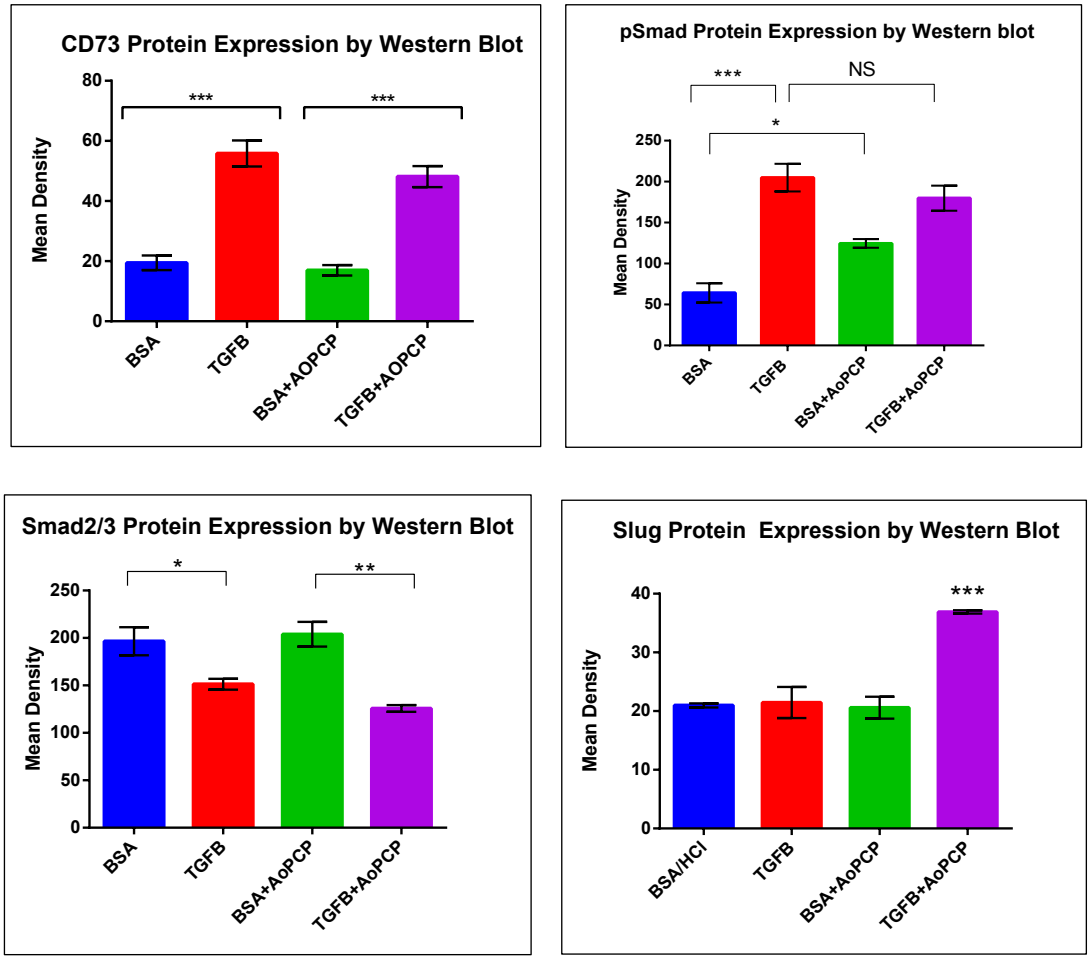

Figure 6. Densitometry for Western blot protein analysis of CD7**3**, Psmad2/3, Smad2/3 and Slug protein expression. Psmad2/3 is phosphorylated at residues Ser465/467. Significance is indicated by *.

Expression of CD73 in HEC-1-A cells was also analyzed using immunofluorescence. Cells were placed in one of four different treatment groups: (1) treatment with BSA/HCl, (2) treatment with TGF-β1, (3) treatment with BSA/HCl+AoPCP, and (4) treatment with TGF-β1+AoPCP. Cells that were treated with TGF-β1 showed increased expression of CD73, even when they were treated with AoPCP, supporting similar western blot results.

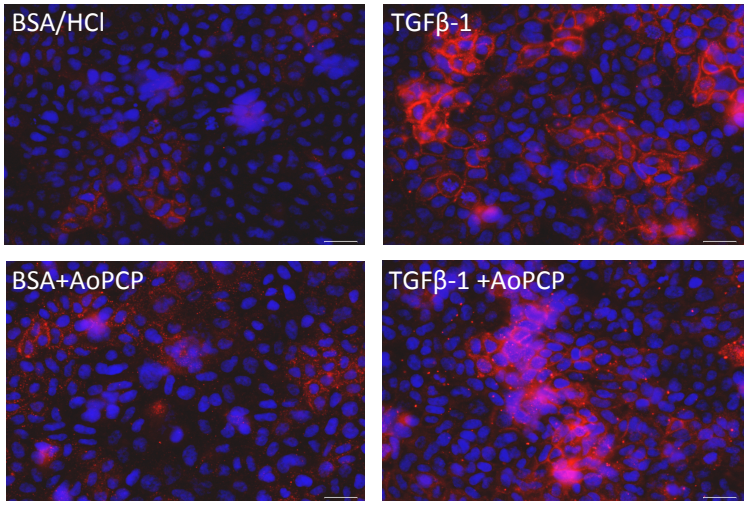

Figure 7: CD73 immunofluorescence expression in HEC-1-A cells. CD73 expression increased when cells were treated with TGF-β1.

Immunofluorescence results for CD73 expression in HEC-1-A cells are summarized in Figure 8. A one-way ANOVA was run to analyze the data. There was a significant difference in CD73 expression between the BSA/HCl and TGF-β1 treatment groups ($p<0.0001$) and the BSA+AoPCP and TGF-β1+AoPCP treatment groups ($p<0.0001$). There was no significant difference in CD73 expression between TGF-β1 and TGF-β1+AoPCP treatment groups.

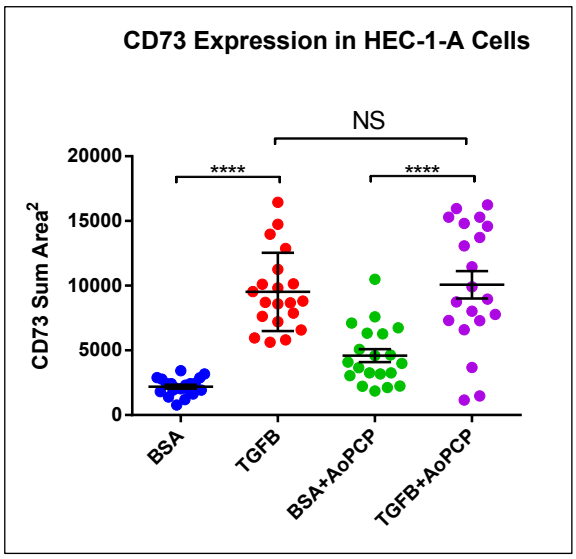

Figure 8: CD73 intensity measured by sum area. Individual markers indicate photographs of the cell plates. There was a significant difference in CD73 expression between cells treated with

BSA and TGF-β1. There was also a significant difference in CD73 expression in cells treated with BSA+AoPCP and TGF-β1+AoPCP.

An MTT assay was performed to measure cell proliferation at 12-hour increments over 72 hours. HEC-1-A cells were placed in one of four different treatment groups: (1) treatment with BSA/HCl, (2) treatment with TGF-β1, (3) treatment with BSA/HCl+AoPCP, and (4) treatment with TGF-β1+AoPCP. Adding TGF-β1 caused a significant decrease in cell proliferation (*$p<0.05$) compared to the BSA/HCl treatment. Cells treated with TGF-β1+AoPCP experienced significantly more cell growth compared to cells treated with just TGF-β1 (#$p<0.05$). Results of the experiment are shown in Figure 9.

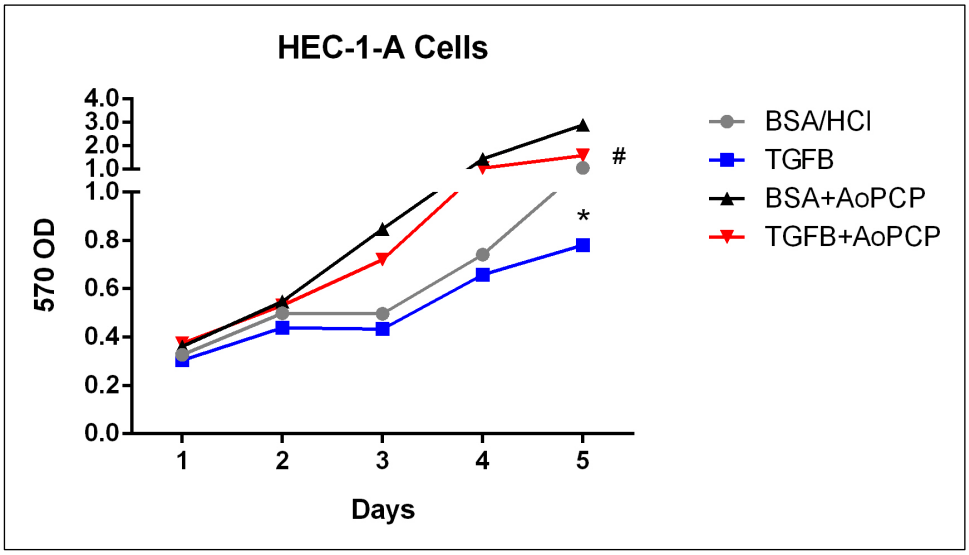

Figure 9: MTT assay results for HEC-1-A cells treated with BSA, TGFβ, BSA+AoPCP, and TGFβ+AoPCP. HEC-1-A cells were treated without 5'adenosine monophosphate (5'AMP), CD73's substrate. *$P<0.05$ BSA/HCl and TGFβ. #$P<0.05$ TGFβ and TGFβ+AoPCP.

Preliminary data: Loss of CD73 decreases TGFβ-mediated differentiation by F-actin expression

A preliminary experiment (n=1) indicated that inhibiting CD73 with AoPCP results in lower F-actin expression in HEC-1-A cells treated with BSA/HCl and TGF-β1. The results of this experiment are shown in Figure 10.

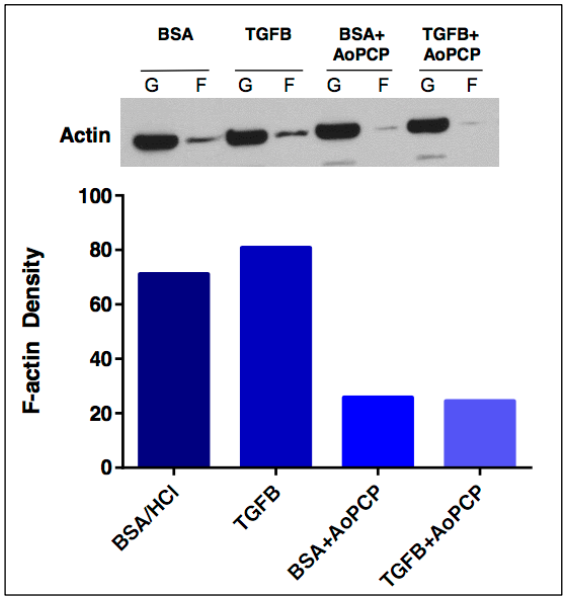

Figure 10: Western blot for G and F-actin and densitometry results for HEC-1-A cells treated with BSA, TGFβ, TGFβ, BSA+AoPCP, and TGFβ+AoPCP. Preliminary data (n=1) shows that F-actin is increased by TGFβ and decreased by CD73 inhibitor, AoPCP.

Discussion

The purpose of this study was to determine if loss of CD73 shifts the TGFβ pathway from acting as a tumor suppressor in normal endometrial tissue to promoting tumor progression in early-stage EC. To better understand this paradoxical behavior of TGFβ, I studied both normal murine endometrial tissue and a model of early-stage EC, HEC-1-A cells. To assess the tumor-suppressing behavior of TGFβ, I studied epithelial differentiation via F-actin expression in the murine endometrial tissue. To assess the tumor promoting activities of TGFβ, I measured protein expression of tumor-promoting gene, *Slug*, and cell proliferation in HEC-1-A cells via MTT assay. *I hypothesized that TGF-β1-mediated increase of CD73 in normal endometrium is correlated with increased epithelial differentiation through increased F-actin expression. I also hypothesized that inhibiting CD73 increases TGF-β1-mediated tumor-promoting activities.*

In normal tissue, the TGFβ pathway is associated with regulating cell cycles by mediating cell growth, differentiation, and apoptosis.[8] Normal epithelial and stromal cells of the endometrium undergo proliferation, migration, differentiation, and death throughout a woman's estrous cycle. TGF-β1 plays an important role in the regulation of these processes.[17] High TGF-β1 expression in the stroma during the secretory phase of the estrous cycle suggests that TGF-β1 has a role in controlling cell proliferation and differentiation.[18] Studies have also shown that TGF-β1 regulates endometrial epithelial growth through its stimulatory effects on the formation of the extracellular matrix (ECM).[17] TGF-β1 may also be involved in regulating matrix metalloproteinases (MMPs), which are proteases involved in endometrial remodeling during the proliferative phase of the estrous cycle, preventing carcinogenesis.[17] Unlike other tumors that tend to have a higher concentration of stromal cells, endometrial tumors tend to grow at a microscopic level of interconnected glands.[1] Therefore, maintaining the ability of TGFβ and its

downstream targets to promote epithelial cell-to-cell adhesions and evade EMT would be considered necessary to prevent EC initiation and progression.

I found that TGF-β1 causes increased F-actin expression in normal endometrial epithelial tissue. Increased F-actin expression was positively correlated with CD73 expression. These findings built upon previous experiments conducted by Dr. Bowser in which intraperitoneal injection of TGF-β1 significantly induced CD73 expression in models of early-stage EC. Previous experiments also showed that CD73 maintains epithelial barrier function by generating adenosine from ATP that is released to the cell surface in the event of tissue injury or disease. In many tissues, CD73 and extracellular adenosine are crucial for maintaining epithelial barrier function and epithelial integrity. In the endometrium, adenosine released into the extracellular space acts on the adenosine A_1 receptor, which induces the polymerization of F-actin necessary for reforming cell-cell adhesions.[11] The results of the present study combined with data from these previous experiments would suggest that TGF-β1 causes an increase in F-actin via CD73. In this experiment, TGF-β1 was still acting as a tumor-suppressor by increasing epithelial barrier function via increased F-actin expression. Maintaining the barrier function of epithelium cells in the endometrium prevents the progression of cancer by preventing invasion and metastases of cancer cells.[19]

When CD73 was inhibited by AoPCP in HEC-1-A cells, treatment with TGF-β1 resulted in the loss of barrier function of epithelial cells, shown by an increase in proliferation and expression of epithelial-mesenchymal-transition (EMT) transcription factor, *Slug*. Although the initial stimulus for EMT is not well known, it is associated with the reduction of cell-cell adhesion molecules, such as E-cadherin, and an increase in the expression of *Slug*.[20] EMT is important for tumor progression because it allows epithelial cells to gain motility and loss of

cell-cell contact. These changes allow cells to more easily invade surrounding tissue and metastasize to distant sites in the body.[21] The expression of *Slug* has been found to be an important factor of EMT, and therefore the invasion and metastasis of endometrial carcinomas.[20]

Inhibiting CD73 in HEC-1-A cells treated with TGF-β1 caused a significant increase in proliferation as compared to cells treated with TGF-β1 alone. However, cells treated with TGF-β1 experienced decreased proliferation compared to cells treated with the control, BSA/HCl. Thus, in the absence of CD73 inhibition, TGF-β1 appears to have acted as a tumor suppressor by decreasing proliferation. When CD73 was inhibited, TGF-β1 increased cell proliferation, acting as a tumor promoter. In a preliminary experiment, inhibiting CD73 in HEC-1-A cells treated with TGF-β1 caused a decrease in F-actin expression, and therefore, epithelial differentiation. These results combined indicate that inhibiting CD73 causes an increase in TGF-β1-mediated tumor-promoting activities, as summarized in Figure 11.

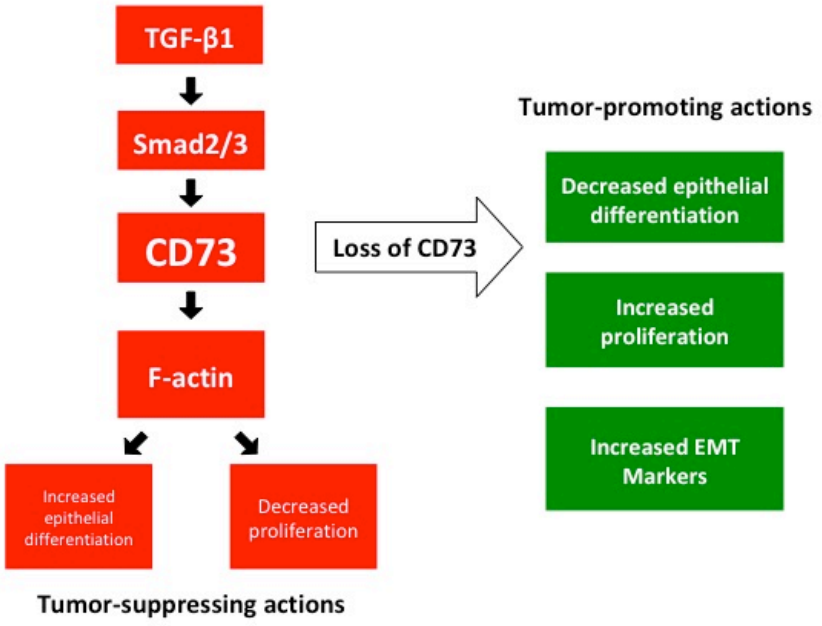

Figure 11: The TGFβ pathway normally functions as a tumor suppressor by increasing epithelial differentiation and suppressing proliferation. The loss of CD73 appears to cause TGFβ to become a tumor promoter, by decreasing epithelial differentiation, increasing cell proliferation, and increasing expression of EMT pro-metastatic transcription factor, Slug.

Future experiments can focus on determination of the role of CD73 in TGF-β1-medicated increase of F-actin. Previous research has shown that CD73 localizes to areas of cell-cell contact in low grade, well-differentiated endometrial tumors.[1] This localization of CD73 could be compared to F-actin expression in normal endometrium to determine if CD73 is causing the increase of F-actin expression, and therefore, differentiation. Another approach could be to inhibit adenosine production by CD73 using adenosine receptor antagonists. If treatment with TGF-β1 resulted in reduced F-actin expression, it would provide evidence that CD73 is involved in this pathway.

The tumor-suppressing functions of TGF-β1 could be further validated in similar experiments by analyzing the expression of E-cadherin and $P27^{Kip1}$ in mouse uteri. E-cadherin is a cell-cell adhesion molecule found downstream in the TGFβ pathway involved in suppressing tumor development.[22] E-cadherin is commonly used as a marker for epithelial tissue, but its presence could also be used to assess the effects of TGF-β1 on barrier function in endometrial tissue. $P27^{Kip1}$ is a cyclin-dependent kinase inhibitor found downstream in the TGFβ pathway that functions to stop cell-cycle progression in the G1 phase. TGF-β1 has been found to increase expression of $P27^{Kip1}$ in normal tissue and well-differentiated tumors.[8] Therefore, if TGF-β1 suppresses carcinogenesis in normal endometrium, both E-cadherin and $P27^{Kip1}$ expression would be expected to increase in tissue treated with TGF-β1.

There were several limitations to this study. I was only able to work in the lab for 10 weeks during the CPRIT/CURE Summer Research Experience. Another limitation is that TGFβ knockout mice and SMAD knockout mice are lethal, so injecting mice with TGF-β1 is the best way to study TGF-β1's association with CD73. Immunofluorescent staining of mouse uteri with

CD73 resulted in too much background staining, making it impossible to assess positive expression.

The results of this study may improve the understanding of the paradoxical behavior of the TGFβ pathway in endometrial cancer. Development of a treatment that targets and prevents the loss of CD73 may allow TGF-β1 to continue functioning as a tumor suppressor and prevent its development into a tumor promoter. Preventing the loss of CD73 in endometrial cancer may prevent metastases and invasion of tumor cells by maintaining the anti-proliferative effects of TGF-β1. These findings may contribute to the development of a targeted therapy for endometrial cancer. Targeted therapies for many cancers, such as breast cancer and leukemia, result in improved outcomes and prognoses for patients.[23] There are no current targeted therapy options for EC. Since surgery alone is not a definitive cure for advanced-stage EC, research on molecular targets, such as the research presented in this thesis, is needed to develop targeted therapies to prevent the spread of microscopic metastases and recurrences of disease. Although TGF-β1 and CD73 may be potential targets for drug design, therapies that inhibit the TGFβ pathway may have unknown and unintended negative effects. The TGFβ pathway is involved in regulation of the estrous and menstrual cycles, so targeting the pathway may cause unintended side effects in the reproductive cycles of women. There is currently no mechanism to limit the targeted therapies to cancer cells, and there is no way to predict the compensation mechanisms that tumor cells may use. Additionally, it is important to recognize the delicate balance of TGFβ's tumor-suppressor/tumor-promoting activities and their timing in various types of cancer. Therefore, targeting the TGFβ pathway should be studied in the context of specific cancers.

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